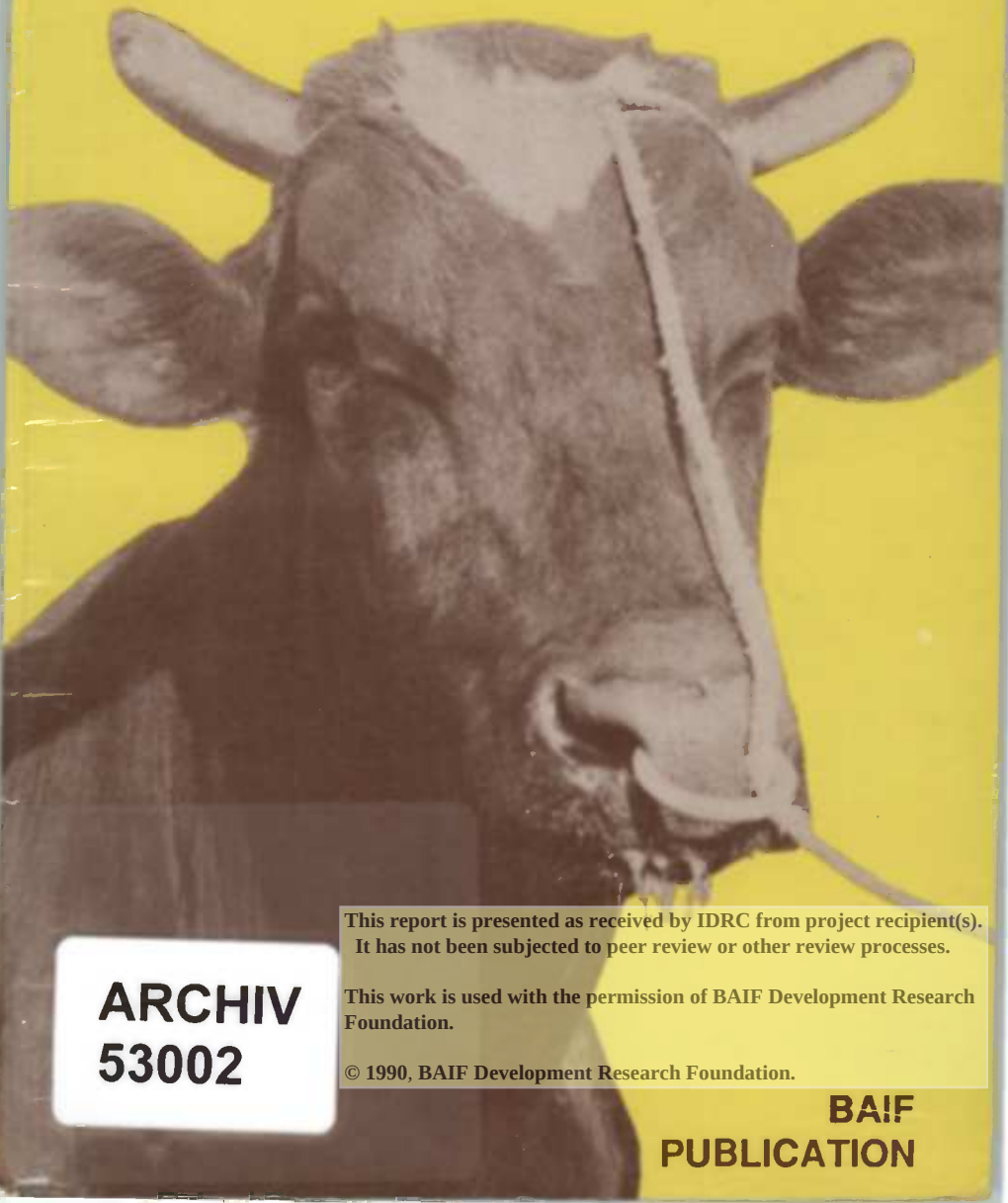
SEMEN PRODUCTION AND ARTIFICIAL INSEMINATION

DR. M. R. BHOSREKAR



BAIF
PUBLICATION

Semen Production and Artificial Insemination

DEDICATED TO

DR. MANIBHAI DESAI

WHO CARRIED THE BENEFITS OF
FROZEN SEMEN TECHNOLOGY
TO THE DOORSTEPS OF
INDIAN FARMERS.

Semen Production and Artificial Insemination

BY
DR. M. R. BHOSREKAR

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CONTENTS

FOREWORD

Livestock is one of the natural resources, well distributed among different income groups in rural India. Among them cattle is the most important animal maintained by almost all the communities for milk, manure and energy. However, majority of our cattle are non-descript, having low milk yield, long dry period and thus uneconomical to the owners.

In the 1960s, the Government of India introduced the programme of cross-breeding to upgrade these non-descript cows using exotic bull semen through artificial insemination. The approach was pragmatic and the impact was miraculus. Cross breeding of cattle was found to be the ideal method to bring the socio-economic transformation among the rural poor within a short period of 3 - 5 years. Availability of super quality semen and correct techniques of AI are the critical factors responsible for the success of the cattle development programme.

Dr. Manibhai Desai, a disciple of Mahatma Gandhi, while working with a Gir herd and their crosses with the exotic breeds at the Nature Cure Ashram realised the potentials of cross-bred cows as well as the weaknesses of available infrastructure in the country. He then established BAIF in 1967 to launch a massive cattle development programme to upgrade the local cattle, by providing the service at the doorsteps of farmers. Production of superior quality semen was taken as an important function of this programme and Dr. M. R. Bhosrekar was assigned this responsibility. A recipient of the Rafi Ahmed Kidwai Award for his work on Buffalo semen freezing, Dr. Bhosrekar brought the latest technology and rich experience acquired at different international and national institutions to set up the Semen Freezing Laboratory at Urulikanchan, which annually produces more than two million doses of frozen semen of international standards.

It is heartening to note that he is sharing his vast knowledge and rich experience in the form of this book, which can serve as an ideal reference manual for Veterinarians, Livestock Development Officers and a textbook for the Animal Science students. I congratulate him for his brilliant work and hope to see many such scholarly publications for the cause of livestock development in the country.

March 25, 1990.

N. G. HEGDE,
Vice President, BAIF

Author's Mind

Some of my friends and well wishers have encouraged me to revise my book and make it up to date so that it can be useful for the graduate students of Veterinary Sciences/Animal Sciences, more so for the post graduate students in the faculty of Animal Reproduction.

This revised edition contains additional chapters on the laboratory tests for quality control of semen, maintenance and care of laboratory equipment. Utilisation of liquid chilled semen for artificial insemination, Breeding management, Oestrus Synchronisation and preservation and utilization of Buck's semen for Artificial Insemination.

Recent advances in the subject have been thoroughly reviewed and literature is added upto 1989.

I sincerely hope that this book will be found most useful for the students of Veterinary and Animal Sciences; persons engaged in semen processing and freezing laboratories, persons engaged in training and teaching of students at different levels, as well as those operating AI programme in the field.

I take this opportunity to thank Dr. Manibhai Desai, President, BAIF Development Research Foundation, Mr. N.G. Hegde, Vice President, Dr. B.R. Mangurkar, Research Programme Organiser, Shri M.P. Marathe and Dr. D.S. Gorhe, Vice Executive Presidents of BAIF Development Research Foundation and Dr. P.A. Deore for their unstinted support to bring out this publication.Special thanks are to Dr.E.S.E. HAFEZ for his permission to adopt the figures from his book.

INTRODUCTION

Probably more has been written and thought about reproduction than any thing else. The first comprehensive treatise on reproduction was Aristotle's "Generation of Animals". After about 200 years another valuable comparable publication appeared in 1910 by Marshell "Physiology of Reproduction".

As early as 1780 the Italien priest Spallanzani attempted to answer the question whether the spermatozoa were actually fertilizing agent for the egg. Dumas (1925) provided definite proof by experimenting on rabbits that spermatozoa are fertilizing agents. Spallanzani did show the possibilities of Artificial Insemination for the first time. After the initial work by Spallanzani only sporadic attempts were made to put the discovery into practice until beginning of the century when Russian Scientist Ivanov (1907) began to investigate the possibilities of collecting, diluting and processing of semen. He started his work with horses and extended it to other domestic species. Milavanov (1938) mostly worked on dilutors for semen.

The first artificial insemination cooperative was organised in Denmark by Sorensen and Gallingholm in 1936. Simultaneously in 1937 and 1938 successful experiments in Artificial Insemination were made in Minnesota, U.S.A. on beef cattle. In 1938 Perry organised the first American Cooperative in New Jersey. Simultaneously in India Sampatkumaran who was trained in U.S.A. started Artificial Insemination in Palace herd of Maharaja of Mysore. In 1942 four

Artificial Insemination Centres were established one at I.V.R.I., Izatnagar and rest were at Lahore, Bangalore and Nagpur. In 1955 a F.A.O. team headed by Prof. Nils Lagerlof visited India to advise on organisation of Artificial Insemination and rural development. "Key Village Scheme". Government of India established Key Village Centres in community development blocks to develop the breed through selective breeding and artificial insemination and other package of practices to support this scheme. Not satisfied with slow progress in improvement of milk production, Government of India under the expertise of Animal breeders introduced Crossbreeding Programme as a National Policy through Intensive Cattle Development Projects. National Dairy Research Institute was the first to experiment with frozen semen in 1961. Thereafter number of organisations came up and now semen freezing and breeding through frozen semen has been adopted at Indo-Swiss Project Munar, Matupatti Kerela, Indo-Swiss Project Patiala, (Pb) Cross-breeding Project Haringata, (WB), Madras Veterinary College, Madras, National Dairy Research Institute, Karnal, and its branch at Bangalore, Indo-Danish Project in collaboration with Government of India at Hissargatta (Bangalore), Indo-Danish Project in collaboration with the BAIF Development Research Foundation, Uruli-Kanchan (Maharashtra). Government of Denmark has taken up six other centres for establishing frozen semen banks in collaboration with State Governments.

With the advent of deep frozen semen technology which scores over the chilled liquid preservation of semen. It is hoped that in the near future frozen semen technology will be adopted all over India for breeding purposes for all species of Livestock. All state governments have adopted artificial insemination for cattle breeding and to certain extent for buffaloes.

CHAPTER I

ANATOMY AND PHYSIOLOGY OF REPRODUCTIVE ORGANS

A knowledge of the male reproductive organs and their functions is essential for persons engaged in management of bulls, collection and handling of semen for artificial insemination. Figure 1 shows the location of different reproductive organs of bull. The reproductive organs of bull can be divided into primary and secondary or accessory organs and external genital or copulatory organs.

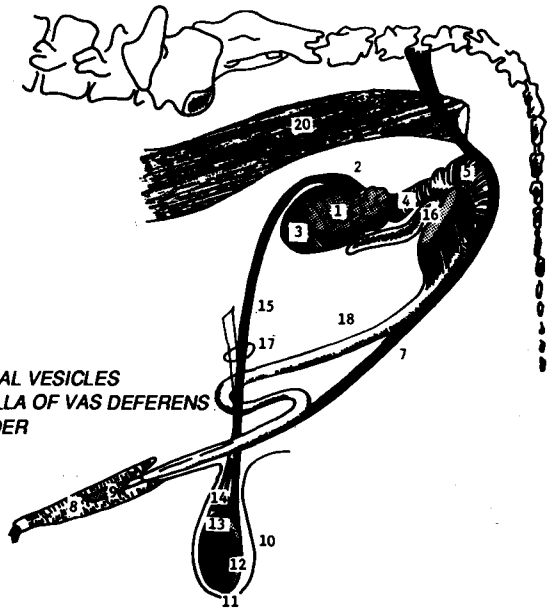
Primary Reproductive Organs	Accessory sexual glands and ducts	External genital organ
1. Testicles	1. Cowper's glands 2. Seminal vessicles 3. Prostate gland 4. Urethral glands 5. Epididymis 6. Vasa-deferens 7. Ampullae	Penis

The Testis

The testicles are two in number, which are carried, out side the body wall in the scrotum. The testicles have at least two functions. (1) The production of Spermatozoa, is the primary function. (2) The production of endocrine substances which markedly affect the development and behaviour of the male, is complementary function.

In the bull the testicles are elongated ovoid organs placed with their long axis vertical in the scrotum. In

THE GENITAL ORGANS OF THE BULL



1. SEMINAL VESICLES
2. AMPULLA OF VAS DEFERENS
3. BLADDER
4. URETHRAL MUSCLE SURROUNDING PELVIC URE THRA.
5. BULBOSPONGIOSIS MUSCLE
6. ISCHIOCAVERNOSIS MUSCLE
7. RETRACTOR PENIS MUSCLE
8. PREPUTIAL MEMBRANE AND CAVITY
9. GLANS PENIS
10. SCROTAL SAC
11. TAIL OF EPIDIDYMIS
12. BODY OF EPIDIDYMIS
13. HEAD OF EPIDIDYMIS
14. SPERMATIC CORD
15. VAS DEFERENS
16. ISCHIAL ARCH
17. INGUINAL CANAL
18. PENIS
19. SIGMOID FLEXURE
20. RECTUM

Fig No. 1

FIG 1

the mature animals they are 12-16 cm in length and 6 to 8 cm in breadth. Each testes weighs 300 to 500 g including epididymis depending upon the age, body weight and breed of the bull. Normally the testis in the bull are equal in size have a firm but not hard consistency and can be freely moved up and down with in scrotum. In buffalo bulls the size of the testicles is slightly different they are smaller 10 to 12 cm in length, 5 cm in width and 3 to 4 cm in thickness and weighs about 100 g. each.

The testicles are made up of connective, interstitial and spermatogenic tissue. They are richly vasculated and have an intricate system of nervous tissue. The sperm forming tissue is found in semeniferous tubules. These tubules are highly convoluted with tremendous surface area. Laid end to end the tubules in one testicle of the bull would be some 10000 to 15000 feet in length. (Fig. 2). In the interstitial tissue leydig cells are present, which are responsible for secretion of male hormones, which are intrinsically linked with the production of seminal plasma.

The wall of the seminiferous tubules consists of a basement membrane and a multilayered sperm producing epithelium. This epithelium consists of two types of cells; a) Germ cells differing in age and morphology, generally arranged in concentric layers. b) Sartoli cells which are slender pillar like structures perpendicular to the basement membrane, to which they are attached by a flat base. They are situated among the densely crowded germ cells to support and nourish them. (Fig. 3)

The seminiferous tubules pass into the body of the mediastinum and unite with a net work of ducts, the

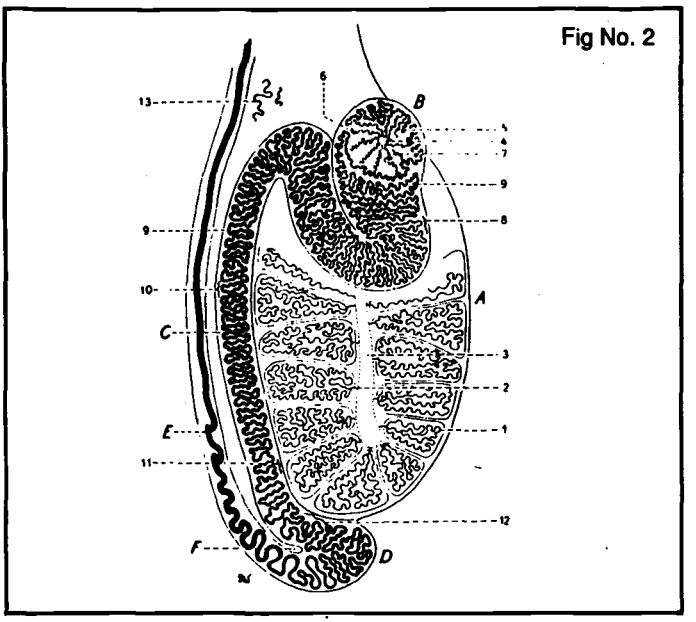


Fig. 2 : Schematic drawing of the tubular system of testis-epididymis in the bull (for clarity the duct system of rete testis is omitted).

A, Testis; B, head of epididymis; C, body of epididymis; D, tail of epididymis; E, deferent duct;
F, site of ligamentum testis.

1) Lobule with semiinferous tubules; 2) tubulus rectus; 3-4) rete testis; 5) efferent duct; 9) epididymal duct; 6,7,8,10,11,12,13) blind ducts and duct rudiments from where cyst formation may eventually occur. Blom & Christensen 1960. Nord. Vet. - Med., 12, 453).

retetestis, which is lined with a cubic epithelium. At the proximal end of the testes the net work of rete passes through a 4 to 5 cm wide opening in the tunica albuginea and connects with the epididymis by the 13 to 15 tubules constituting the ductuli efferentes testis.

Between the seminiferous tubules there is loose connective tissue containing blood and lymph vessels, nerves and isolated groups of polygonal interstitial cells (leydigcells) with large spherical nuclei :

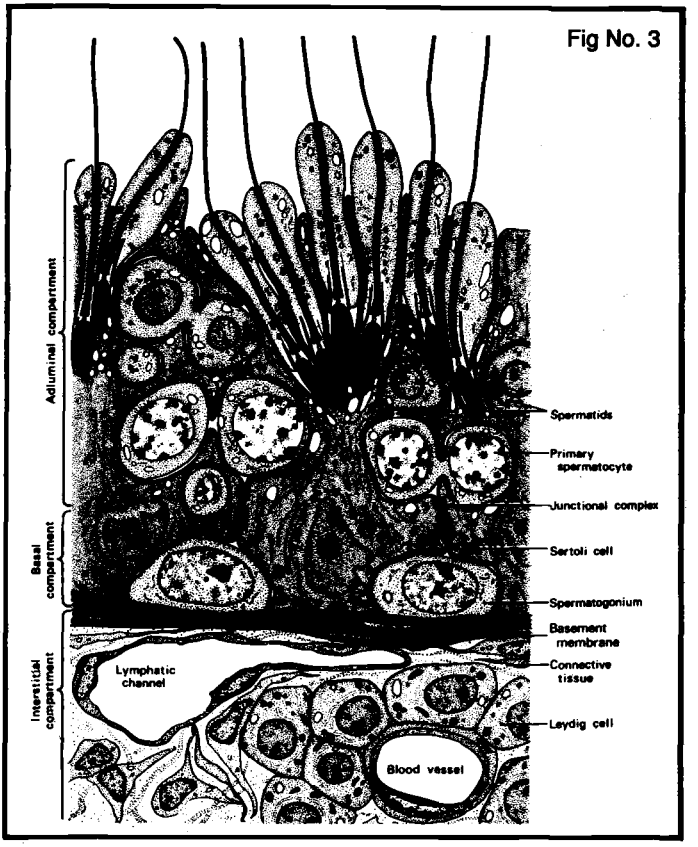


Fig.3 : Drawing of part of a seminiferous tubule showing the relationship of the germ cells to the adjacent Sertoli cells. Formation of spermatozoa starts near the basement membrane when a spermatogonium divides to form other spermatogonia and ultimately primary spermatocytes. The primary spermatocytes are moved from the basal compartment through the junctional complexes between adjacent Sertoli cells into the adluminal compartment where they eventually divide to form secondary spermatocytes (now shown) and spherical spermatids. The spermatogonia, primary spermatocytes, secondary spermatocytes, and spherical spermatids all develop into space between two or more Sertoli cells and are in contact with them. During elongation of the spermatid nucleus, the spermatids are repositioned by the Sertoli cells to become imbedded within long pockets in the cytoplasm of an individual Sertoli cell. When released as a spermatozoon, a major portion of the cytoplasm of spermatid remains as a residual body within a pocket of the Sertoli cell cytoplasm. Note the intercellular bridges between adjacent germ cells in the same cohort or generation.

Testicular tissue has prolific activity : In the bull 1 g of testicular tissue manufactures on an average 9 x 10^6 sperm per day (Willet and Ohms, 1957) i.e. 6000 per minute. In some mammals the testis and accessary sex organs function fully during the breeding season. In farm mammals sperm production is maintained through out the year with some seasonal variations.

The Epididymis

Attached to each testicle is a intricately convoluted tube the epididymis, which extends down the outside of the testicle to its base. The epididymis consists of three parts caput, corpus and cauda.

Histology : The 13 to 15 efferent ducts occupy about 1/3rd of the caput of the epididymis. They have a diameter of 100 to 300 microns, contain only a few sperm in the lumen and a very characteristic epithelium. Two types of cylindrical epithelial cells are found attached to the thin basement membrane of the ducts (a) Secretary cells with large cytoplasmic granules and (b) Ciliated cells with kinocilia, motile cilia, all beating outwards. Blom (1944) has found that these ciliated cells normally detach their ciliated borders and excrete them in to semen at the rate of one per 10,000 sperm (i.e. about 500,000 in an average ejaculate).

Near the centre of the caput epididymis the epididymal duct starts its long and tortuous course through the rest of the epididymis. The epididymal duct can be differentiated into 6 distinct regions histologically and cytochemically. The Lumen of the ducts is 1 mm in diameter.

Functions : The epididymis has four major functions. 1. Transport, 2. concentration, 3. maturation and 4. storage of sperm. The functions of epithelium is in part absorption and in part secretion. The epididymis behaves like Kidney by virtue of its embryonic characteristics.

1. Transport : After their release from the seminiferous tubules. The sperm pass rapidly into the epididymal duct proper via the tubuli recti, rete testis and ductus efferentes. From rete testis to efferent ducts sperm pass by fluid pressure in the testis.

Their passage through the efferent ducts is assisted by the active onward beating cilia of the cilliated cells and in the epididymal duct by the peristaltic movement of the musculature of the wall. The transport of the sperm from the germinal epithelium to the cauda of the epididymis takes 7 to 9 days in the bull to some degree depending upon the frequency of the ejaculation, (Knudson, 1954). The transport is also assisted by the continuous flow of fluids from the testis.

The epididymal duct is a single unbranched, strongly winding canal, in bulls upto 50 mts. long. Transport of sperm along the duct is brought about by spontaneous rhythmic contractions in the layer of smooth musculature surrounding the duct, while the sperm remain in a quiescent state.

Contractions in duct are dependent on androgen hormones. The contractions are regulated via autonomous nervous system but the reaction to nervous stimulation is not similar along the duct.

Oxytocin which is released in connection with the sexual preparation of the male and at ejaculation will aid in strengthening the muscular contractions.

2. Concentration : From the dilute sperm suspension originating in the testis, water is absorbed into the epithelial cells during the passage through the epididymis especially in caput and a highly concentrated suspension (4000000 or more/mm^3) is left in the tail of the epididymis.

3. Maturation : The physiological maturation of sperm concerns their morphology, their biochemical and functional characteristics. When the sperm leave the testis they are provided with a cytoplasmic remnant (derived from Golgi complex) situated at the neck of the tail. (Proximal cytoplasmic droplet) When the sperm have passed the flexure of the epididymal caput and entered into the distal part of the caput, the droplet has moved to the distal part of the mid piece i.e. distal droplet. This maturation is achieved probably as a result of secretions from the epithelial cells.

The membranes surrounding the head are loosely fitting and easily distinguishable in the sperm from epididymal caput (observed by electron microscopy) while the acrosome is very tightly contracted around the head of sperm from the cauda epididymis.

Biochemically epididymal sperms differ in several respects from ejaculated ones, During epididymal transit the sperm become increasingly dehydrated, thus sperm from cauda have a higher specific gravity than those taken from the caput. Furthermore the spermatozooal content of phospholipids decreases as the sperm move from caput to cauda.

It is well established that sperm from testis and upper parts of the epididymis are immobile and cannot be made mobile. The ability of the flagellar motility of the tail is acquired gradually during epididymal transit and only sperms from cauda are capable to show progressive motility similar to that of ejaculated sperm.

The development of the fertilizing ability is also gradually acquired during the epididymal passage. Sperm from the caput are not able to fertilize ovum, while cauda sperm exert a normal fertility. Extensive research for last 10 to 15 years has substantiated that the major factor in the maturation of sperm can be ascribed to a very active function of the epididymal epithelium.

The secretary function of the epididymis is limited to a very few compounds. The most important of which is glycero phosphoryl choline which is present in fairly high amounts in the semen of both mammals and poultry. The much more conspicuous characteristic of the epididymis is its large number of cells typical for epithelia engaged in absorption phenomena such as epithelium in proximal tubules of kidney.

The ability to absorb is not limited to fluid and electrolytes but also high molecular substances such as hormones (Testesterone) and other proteins.

The epididymal fluid has a composition which could effect keeping the sperm immotile and reducing their metabolic activity to a minimum within the duct (Physiological anabiosis). The general features of epididymal plasma is as follows :

1. Low pH (around 6)
2. High partial Co_2 pressure
3. Lack of metabolizable substrate
4. High osmotic pressure
5. Relatively high $\frac{K+}{Na+}$ in Cauda
6. Inhibitory effect on metabolism by testosterone.

To these factors may be added that sperm are densely packed within the duct. These conditions help in conserving the life span of sperm in the duct (5-6 weeks) at a temperature only about 3°C below the body temperature.

4. Storage : The tail of epididymis is the sperm storage depot. The concentration of the sperm is very high and the lumen of the duct is relatively wide. It is not surprising, therefore, to find that half of the total number of sperm are stored in this part. (Baily & Smith, 1958) which constitutes only about a quarter of the length of the epididymal length. The conditions in the tail are optimal for preserving the viability of sperm which are in quiescent state of metabolism. The sperm will remain alive and fertile till 60 days even if the epididymis is legated. The fact that the spontaneous rhythmic contractions of the epididymal wall do not involve the cauda (except during sexual excitement) makes this compartment particularly suited for storage of sperm.

While the sperm of a similar age are constantly carried into the cauda, this part of the duct will contain sperm of varying age as mixing of sperm inside cauda also takes place.

The size of the sperm stock is regulated in the first time by ejaculation, besides sperm are passed without ejaculation via excretory ducts.

During sexual preparation of the bull sperm are carried from the distal cauda due to contractions in the wall into the ductus deferencs the so called emission. The better the bull is prepared the larger number of sperm will be expelled at ejaculation.

The mixing of sperm with seminal plasma takes place in the pelvic part of genital tract.

During extremely long periods of sexual rest, the first few ejaculates may contain non fertile sperm. Detachment of acrosome cap (galea capitis) is one of the first changes visible in the sperm (Blom 1945).

Leading upward from the epididymis are the vasa deferentia (Fig. 2). These are slender tubes connecting with the urethra. The vasa deferentia enlarge to from the ampullae. The ampullae are located just above the exterior part of the pubis,where they join and progress forward as the urethra. Sperm are stored in the ampullae until the time of ejaculation.

Lying on either side of the ampullae are the seminal vesicles. They are also double glands. They are about 2 to 3 inches long and approximately one inch wide. These are lobulated organs and secrete seminal plasma. These seminal vesicles empty into ampullae (Fig. 1). Cowper's gland are also two in number located on either side of the urethra. These glands are deeply embeded in bulbo urethral muscle (Fig. 1). It also secretes seminal plasma, which serves as a carrier for spermatozoa. Prostate gland is a single

gland and is ring shape, located near the neck of the bladder, surrounding the urethra. The secretion of this gland is alkaline in nature, it also adds to seminal plasma.

In addition to these, there are glands located in urethral musculature called urethral glands.

Penis has the function of draining the bladder as well as it serves as a copulatory organ to introduce spermatozoa into vagina in natural course.

Spermatogenesis

A. Conditions for proper spermatogenesis

Spermatogenesis goes on in testis much below body temperature (at least 3°C. to 4°C. less than body temperature). Only exception in mammals is Elephant. The placement of testis is different in different species. In bulls, buffalo bulls, rams and bucks the testis are suspended in vertical plane in the inguinal region. The testis of horse are suspended in horizontal plane parallel to body in the inguinal region. The testis of boars, camel are placed posteriorly below anal region in inclined plane in the scrotum.

Scrotum

The scrotum is a pouch containing the testis. In relaxed state the scrotum of the bull is bottle shaped. It has a light coat of hair. Beneath the skin is a tunica dartos composed of smooth muscle fibres with collagenous and elastic connective tissue. It surrounds both testis and also forms a partition between the two

halves of the scrotum. The next layer is of white dense tunica vaginalis communis surrounding two halves of the testis.

Functions :

It serves a regulatory function of maintaining temperature of the testis and epididymis 3° to 4°C lower than that of the body. This is accomplished by a double muscle system. The external cremaster muscle and tunica dartos. Exposure to cold make the dartos to contract thus causing the scrotal skin to pucker and wrinkle shortening the scrotum and thus forcing the testes closer to body wall.

In hot climate, the dartos is relaxed causing the wrinkles to vanish. The surface area is extended. The external cremaster suspends the testis by relaxing in scrotum. The thermoregulatory mechanism of the scrotum is important for normal sperm production in almost all mammals.

In very hot weather particularly if there is no shade the mechanism may breakdown and cause degenerative changes in the germinal epithelium. The thermo regulatory mechanism is achieved by cooling the testicular venus blood during its course near the scrotal skin and then transferring this coolness to the incoming testicular arterial blood in the spermatic cord which acts as a counter current heat exchanger. The scrotal skin is kept cool by abundant population of sweat glands and is also well endowed with temperature receptors which evoke powerful physiological responses in the animal if the scrotum is warmed. The blood supply to the testes is through a single artery running straight to caudal part of the

testes along the epididymis coming up through anterior border and giving off branches reaching paranchyma with minimum branching. There are coils and arches along side the rete testis and then turn back and begin to branch to supply to paranchyma.

The purpose of this peculiar anatomy is still unknown but it most probably helps to eliminate the pulse pressure. Blood flow through the testis is comparatively low so that the testicular venous blood is normally only about 50% saturated with oxygen.

B. Spermatogenesis

Spermatogenesis is the process by which sperm are formed in the seminiferous tubules of the testes. Sperm producing capacity of the testes is predetermined by heredity. Spermatogenesis is commenced at puberty i.e. when the animal is sexually mature. During puberty the tubules acquire lumina and the germinal epithelium changes from a simple to a complex characteristic of the sexually mature male. Spermatogenesis will normally continue throughout life until senelity sets in when progressive atrophy of tubules occur and only a few are capable of producing sperm.

Wild species breed only at certain seasons and the cycle is usually dependent on the day length. In such animals the testis regress completely during the non breeding season and the germinal epithelium returns to the state of the young sexually immature male. In farm animals seasonal breeding is not clearly defined. The testis usually remain in the scrotum and do not undergo cyclical changes. In bulls and Buffalo bulls seasonal variations are not clearly marked. In buffalo

bulls high environmental temperature quite independently of light will depress the sperm production but it is not known if this is a direct effect on the testis or caused indirectly by depressed secretion of thyroid gland. In most mammals the testis are maintained in the scrotum several degrees below that of the body temperature by contraction and relaxation of cremaster muscle. This lower temperature is essential for spermatogenesis.

Sperm are formed within seminiferous tubules from spermatogonium or sperm mother cells which lie in the basement. The process is complex and involves cell division and differentiation during which the number of chromosomes is halved. Ist phase (spermatocytogenesis) which comprises the cellular division phases and ends with the formation of spermatids. The other phase (spermeogenesis) comprises of series of changes in the spermatids without cellular division and during which the spermatids are differentiated into sperm.

The epithelium of the seminiferous tubules consists exclusively of cells. Besides the spermatogenic cells proper (i.e. spermatogonia, spermatocytes, spermatids) is found a smaller number of sartoli cells (supporting cells, nutritive cells) situated peripherally in the tubules against the basal membranes. Their function is not well understood but they are associated with the metamorphosis intimately.

In histological sections of the testis the spermatogenic cells are arranged in a characteristic pattern in each cross section of seminiferous tubule. The pattern differs from tubule to tubule. If sufficiently large number

of cross-sections are examined it will be seen that the different cellular arrangements will repeat themselves with characteristic frequencies. This complete series of cellular arrangements is termed a cycle of seminiferous epithelium or epithelial cycle.

In most domestic mammals a cycle can be divided in 8 easily diagnosed stages (Fig.4). The cellular events taking place during spermatogenesis can be summarised as follows.

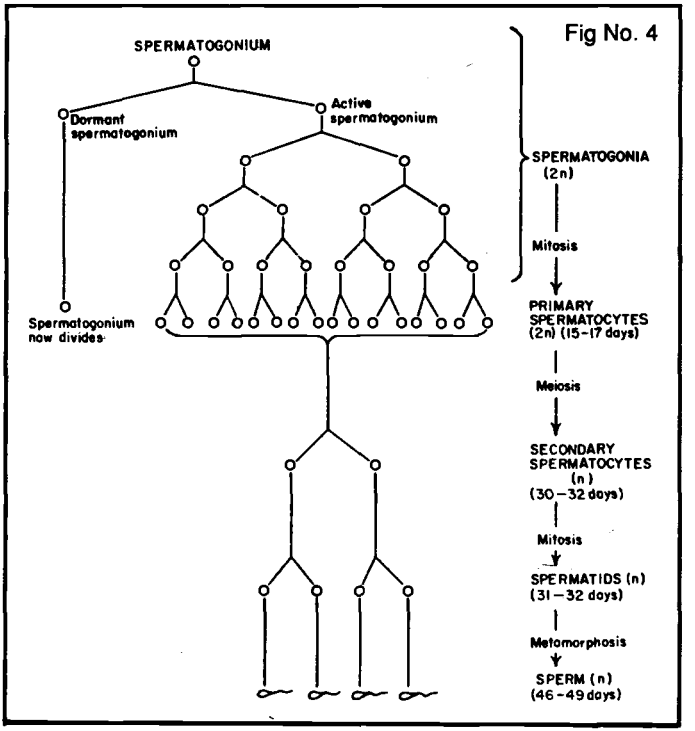


Fig. 4 : Diagram of spermatogenesis in the ram. The sequence of events is probably similar in the bull but may differ slightly in other mammals including the boar and stallion. The chromosome number and the time from the formation of the original spermatogonium are given in brackets. All the primary spermatocytes divide in the manner indicated. (Adapted from Ortavant, 1959. In Reproduction in Domestic Animals, H.H. Cole and P.T. Cupps, edits, Vol.2, New York, courtesy of Academy Press).

From spermatogonia A, two spermatogonia A are formed by mitosis one of this remains undivided for sometime as resting or dormant and functions as mother cell for new generation of spermatogenic cells while the other divides into two intermediary spermotogonia (I. Spermatogonia). These in turn divide each into two B. spermatogonia which finally by division each forms two primary spermatocytes. All these divisions are mitotic.

The newly formed spermatocytes and different types of spermatogonia are placed in the periphery of seminiferous epithelium between the sartoli cells.

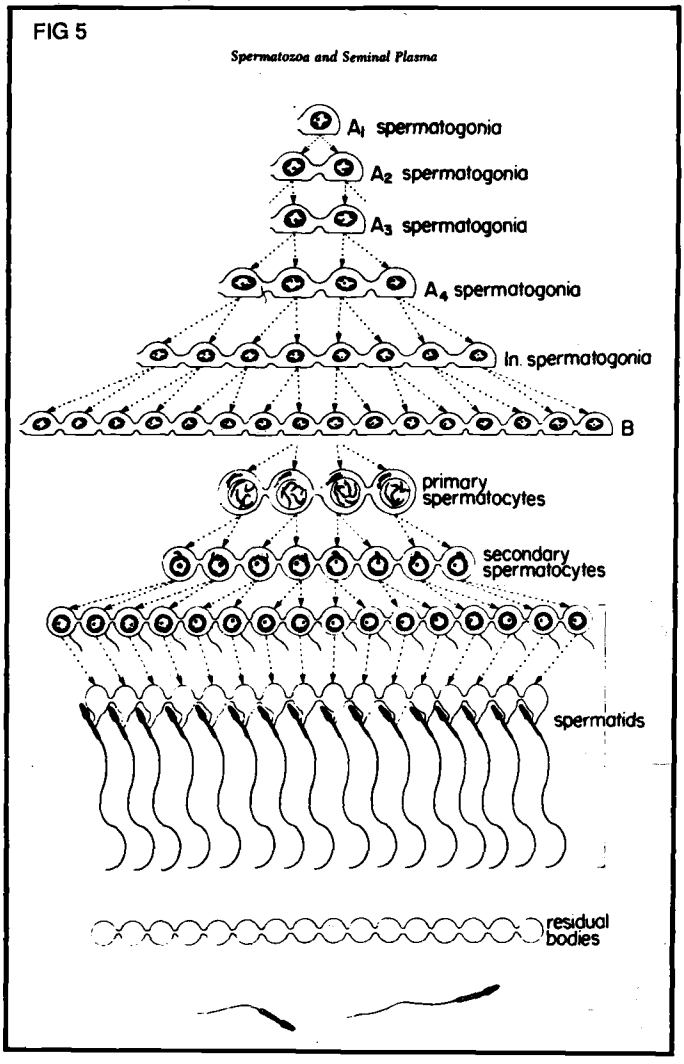
The primary spermatocytes go through the first meotic (or reduction) division (characterised by its long duration) and each gives rise to two secondary spermatocytes which rapidly divide through the second meotic division, each therefore, forming two spermatids containing the haploid chromosome numbers. Finally the spermatids are transformed into sperm (Fig. 5)

The Kinetics of spermatogenic process is shown in the diagram which shows that one mother A. spermatogonium is stem cell for 64 sperm formed and ready to leave the seminiferous epithelium after the lapse of 4.5 epithelial cycles.

Using the isotope technique it is possible to estimate the length of the epithelial cycle. In bull one cycle lasts approximately 13.5 days in Ram it is 10.4 days stallion 12.2 days and in buffalo bulls it is 8.6 days. The period required for A spermatogonium to form sperm is approximately 59-60 days. In buffalo bull this period is markedly less (38 days) that means the epithelial cell cycles are faster as compared to bulls.

Each A3 type spermatogonium produces on an average 27.7 spermatozoa while theoretically 64 number is expected. Hence spermatogenic efficiency in buffalo is 43%. (Sharma and Gupta, 1980).

FIG 5



Based on the quantitative counts of spermatogenic cells in histological sections of the bull testes it has been estimated that one pair of testes in grown up bulls produces approximately 10 billion sperm per 24 hours or approximately 50 million sperm per minute.

Passage of sperm through the epididymis:

Sperm pass quickly from the lumen of seminiferous tubules into the retetestis and via vasa efferentia into the head of the epididymis. Studies using sperm labelled by injecting P_{32} indicate that they take 2 to 3 weeks to pass through the epididymis to tail where they are stored.

Maturation of sperm in the Epididymis :

During their passage through the epididymis sperm are believed to mature or ripen. The cytoplasmic droplet which is attached to the proximal region of sperm midpiece move towards distal end of the mid piece when they reach cauda epididymis. In the ejaculated semen the droplets are completely detached from the sperm. As the spermatozoa move in the epididymis the dehydration occurs and the acrosome cap becomes smaller. The fertilizing capacity increases.

Storage and Ejaculation of Sperm :

Sperm cells survive for longer periods in epididymis. (Salisbury 1966). The fructose is absent in epididymal fluid and G.P.C. is quantitatively more. Sperms are able to utilise G.P.C. as energy source until it comes in contact with the secretions of female tract. It may be

endogenous phospholipid which may be used as a substrate by epididymal spermatozoa as energy source.

C. SARTOLI CELLS

Sartoli cells or nurse cells are the somatic elements of the seminiferous epithelium. It is assumed that their number does not vary in adult and these do not divide. The name of these cells originates from the fundamental discovery of Sartoli who described for the first time the branched cells.

These cells are rich in glycogen, glyco proteins and lipids. They also contain some steriods. The main function of sartoli cells is nutritional and protection to germ cells. They are also concerned in release of spermatozoa.

Other functions consist of :

1. Resorption of residual bodies,
2. Secretion of estrogen and
3. Intermediary for action of gonadotrophins on germ cells

The role of secretion of estrogen is still questionable.

D. ESTABLISHMENT OF SPERMATOGENESIS

The growth rate in first 2 to 3 months after birth is slow and thereafter is more rapid. After about eighth month in calf and 5th months in lamb the growth again slows down. According to Abdel Rouf (1960), Courot (1962) the spermatogenesis starts at 4th month in calf and first sparmatozoa appears at 7th month. In lamb

spermatogenesis starts at three and half months and spermatozoa appear at 5th Month (Courot, 1962 and Sopsford, 1962).

In buffaloes (Deshpande and Janakiraman 1985) noticed the primary spermatogonia at the age of 200 days the cell number increased to 4.3 per tubular cross section at 300 days while it reached 26.7 cells per tubular cross section at 17 months of age. In bulls the primary spermatogonia were first appeared at 60 days. The buffalo bull calves between 450 to 510 days showed spermatids and spermatozoa and fully active leydig cells. So, for maturity a buffalo takes 510 to 600 days i.e. around 20 months as against 12 to 14 months in bulls. In cross-bred (Brown Swiss x local) bull calves the appearance of spermatogonia took 120 days (Raja and Rao, 1982) as against 200 days in buffalo and 60 days in exotic bull calves. According to Aire and Akpokodje (1975) in Bos indicus calves (Fuloni) the primary spermatocytes appeared at 36 weeks (252 days) of age and spermatozoa first at 48 weeks of age. (340 days)

Initiation of spermatogenesis depends upon mainly two factors (1) Development of animal (2) Age. The development of animal is much more important than its age.

E. TESTIS AS A ENDOCRINE GLAND

In addition to the spermatogenic function testis are endowed with endocrine androgenic activity carried out by leydig cells (interstitial cells). The hormonal activity culminates in production of testosterone which determines not only the production of seminal plasma by accessary sex glands but also in developing following characters :

1 Secondary sex characters like development of hump, horns and sound,
2. Sexual desire or libido, and
3. Protein assimilation - for strong musculature and built up in males.

Fig No. 6

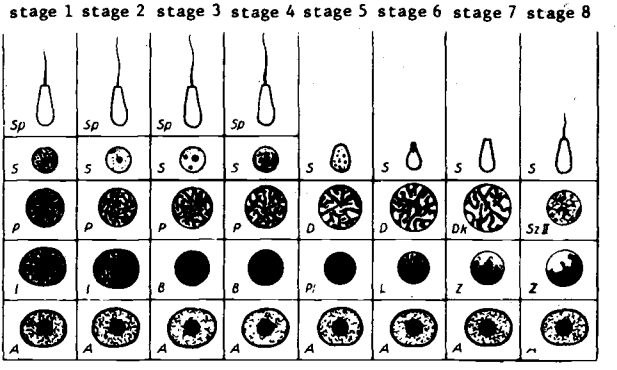


Fig. 6 : Cycle of the seminiferous epithelium in bulls. A = A-spermatogonium, I = intermediary spermatogonium, B = B-spermatogonium.

Primary spermatocytes : P1 = preleptotene, L = leptotene, Z = zygotene, P = pachytene, D = diplotene, Dk = diakinesis, SzII = secondary spermatocyte, S = spermatid, Sp = sperm.

(Adapted from M. Berchtold in F. Schaetz : Die kunstliche Besamung bei den Haustieren, 1963).

Bull's testes was the first organ in which testosterone was isolated for the first time in pure form (David et al 1935) and was shown to be steriod, based on tetracyclic carbon skeleton structure conforming to the formula of 17 ß hydroxy androstane 4 en - 3 one.

In addition to this hormone, bull's testis also donate substantial amount of androstendione, which differs from testosterone in having a ketogroup instead of hydroxyl group. The ratio of androstendione to testosterone in sexually mature bull is 1:10 but in younger animal it is much higher and below 4 months it exceeds 1:1. Bull testis also contain other steriods like androstenol, 17 one progesterone. Stallion testis contain a rich amount of estrogens (Zondak 1934).

ENDOCRINE CONTROL OF SPERMATOGENESIS

The spermatogenesis is under the control of gonadotrophins from anterior pituitary. Hypophysectomy in lamb reduced the weight of testis and the cell number greatly decreased. Injection of I.C.S.H. into hypophysectomised lambs increased the weight of testis. There is no spermatogenesis without gonadotrophins (Courot 1967). (Fig 7 & 8)

Semen and Spermatozoa

The semen is the normal discharge of the male at the time of mating. Its colour ranges from milky white to thick creamy. Its consistency varies from watery to some what thick opaque. Semen is a suspension of spermatozoa in a fluid medium called seminal plasma. Spermatozoa originates from testis the primary sex glands, while seminal plasma is a mixture of secretions from Seminal Vesicles, Cowper's gland, prostate gland, Ampullae, and Epididymis. The amount of semen ejaculated by an adult bull is approximately 2 to 6 ml. The sperm concentration ranges from 800 to 3000 millions per ml. It is influenced by number of factors detailed elsewhere. (Fig.9)

Fig No. 7

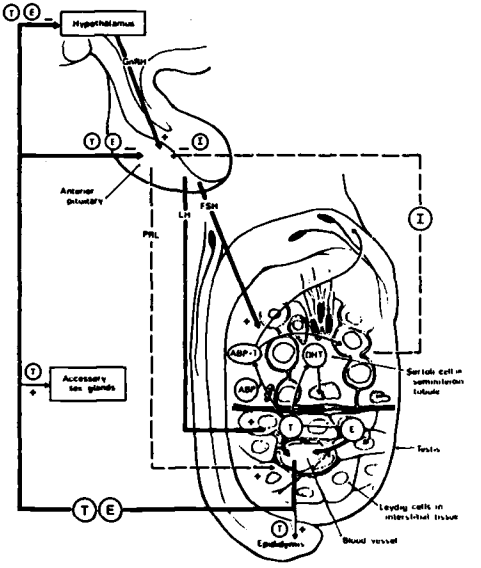


Fig. 7 : Diagram showing interrelationships among hormone production in Leydig cells and seminiferous tubules and the feedback control of gonadal hormones on the hypothalamus and anterior lobe of the pituitary gland. Increased testosterone (T) in peripheral blood, either as a result of increased production by the tests following injection of exogenous hormone, feeds back on the hypothalamus and anterior pituitary to support discharge of luteinizing hormone (LH), and, therefore, the Leydig cells produce less testosterone, and the concentration of testosterone around the semiinferous tubules drops. The control of production of estrogens presumably by the Leydig cells, is not known. The ratio of T to E in blood reaching the anterior pituitary may be associated with differences in the ratio of FSH (follicle-stimulating hormone) : LH secreted by the gonadotrophs. During the infantile period, secretion of gonadotrophin releasing hormone (GnRH) is suppressed so that LH and FSH are secreted in small amounts. Consequently, T is not produced by the Leydig cells, and the Settoli cells remain undifferentiated. During the prepubertal period, pulsatile secretion of GnRH is initiated, the frequency of LH discharges is greatly increased, and testosterone production rises. This is followed by differentiation of Sertoli cells and germ cells and, ultimately, the production of spermatozoa. The role of E, prolac (PRL), and inhibin (I), in adult bulls is unknown. Bovine Sertoli cells produce an androgen-binding prot (ABP) that serves as a carrier of testosterone and may have a role in maintaining androgen concentration with the seminiferous tubules or in providing testosterone to the epithelium lining the proximal portion of the epididymis.

Fig No. 8

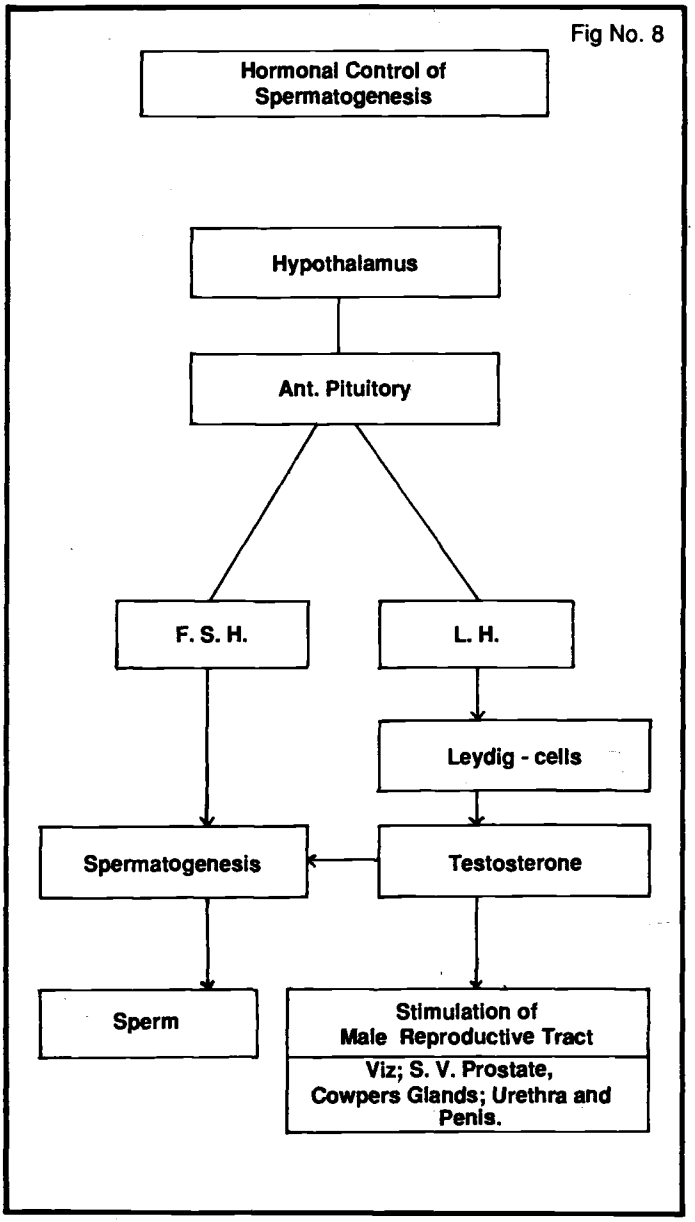

Hormonal Control of Spermatogenesis
Hypothalamus
Ant. Pituitory
F. S. H.
L. H.
Leydig - cells
Spermatogenesis
Testosterone
Sperm
Stimulation of Male Reproductive Tract
Viz; S. V. Prostate, Cowpers Glands; Urethra and Penis.

Fig No. 9

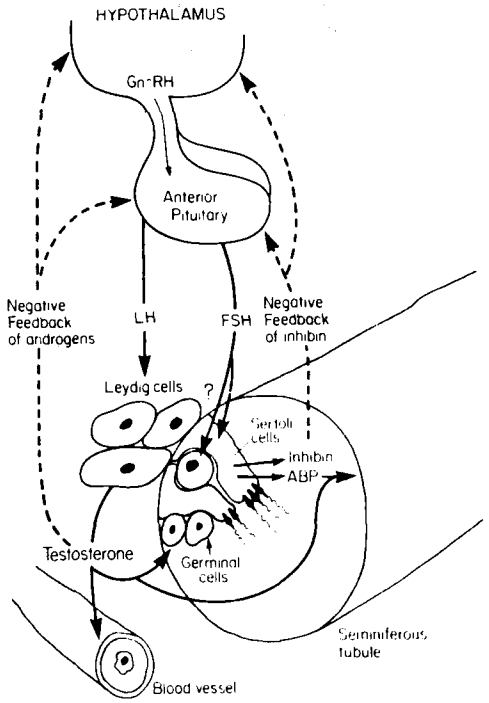


Fig.9 : The endocrine control of testicular function in mammals. The hypothalamus secretes gonadotropin, a hormone-relesing hormone (GnRH), that stimulates the secretion of LH and FSH from the anterior pituitary. The LH stimulates the interstitial cells of Leydig to produce androgens, mainly testosterone. The androgens are secreted into the blood-stream where they cause the development of secondary sex characteristics of the male and development and maintenance of the male reproductive tract. The androgens suppress GnRH, LH and FSH secretion by negative feedback on the pituitary and hypothalamus. Testosterone is also secreted into the seminiferous tubule where it is necessary for maintenance of spermatogenesis. The FSH interacts with receptors on the Sertoli cells to cause production of androgen-binding protein (ABP), conversion of testosterone to dihydrotestosterone and estrogen, stimulation of the spermatocytogenesis, completion of sperm release (spermiation) and secretion of inhibin. The inhibin secreted into the bloodstream has a negative feedback effect on FSH but not on LH secretion. (From Kaltenback, C.C. and Dunn, T.G. (1980) Endocrinology of reproduction. In Reproduction in Farm Animals, 4th ed., E.S.E. Hafez (ed). Philadelphia, Lea & Febiger).

CHAPTER II

SPERM AND ITS FATE

MORPHOLOGY OF SPERMATOZOA

The spermatozoa of the bull is some what tadpole like in appearance and consists of head, neck, middle piece and tail. Total length of bull sperm is 70-80 microns. The head is 8-10 microns long 4 to 5 microns broad and 1 to 1.5 microns thick. The head can be divided in 3 zones. 1) Anterior part, covered by a double walled acrosome cap. 2) Intermediate part or equatorial segment (a small brim covered with a very thin cyto plasmic layer. 3) Posterior part or post equatorial segment, in which the chromatin concentration and staining ability are heaviest. This part is covered by a well developed membrane, the post nuclear cap. The nucleus it self is covered by a nuclear membrane. The base of the head is concave, the implantation groove) to which the tail is fastened. The neck is 1 micron long. The tail consists of i) Mid piece 10 microns 2) The main piece 50 microns and end piece 3 microns long (Table 1).

Anterior centriole is placed proximally against the base of the head where the neck is implanted. The axial filament emerges from the anterior centriole and proceeds through the entire length of the tail. The axial filament is built by fibres of contracttile protein (20), 2, central fibres and 2 rings, each with nine fibres. The outer ring consists of the course fibres, the inner one of double tubular fibres. The posterior centriole consists of a proximal part (small "grains" in the proximal end of the mid piece) and a distal part formed as a ring at a distal end of the mid piece

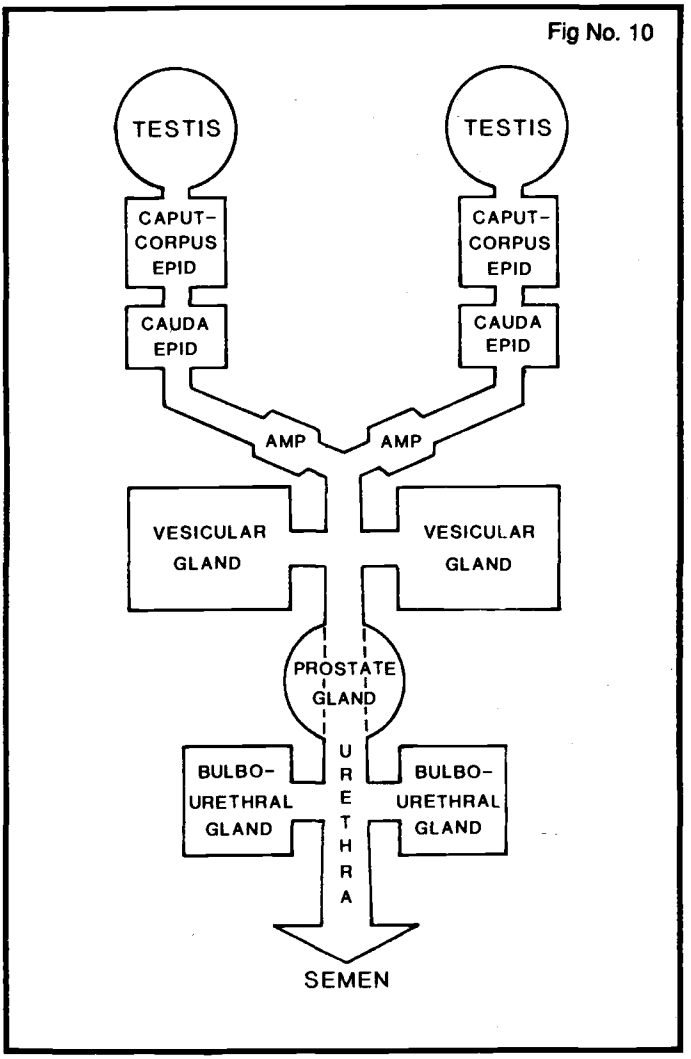


Fig. 10 : Ejaculated semen of most farm animals is composed of, in addition to a small amount of testicular fluid, contributions from several accessory organs including the epidiymis (CAPUT-CORPUS EPID AND CAUDA EPID), ampullary glands (AMP), vesicular glands, prostate gland and bulbourethral glands. The relative contribution of the glands varies not only among sepies but also among individuals within a species and among ejaculates from the same animal.

(terminal ring or Jensen's ring). The mid and main pieces are covered by a fibrous sheath (lipo protein), which is thickest around the mid piece and forming the mitochondrial helix. The end piece contains only the central and inner ring of 9 fibres. The whole sperm cell is covered by a plasma membrane.

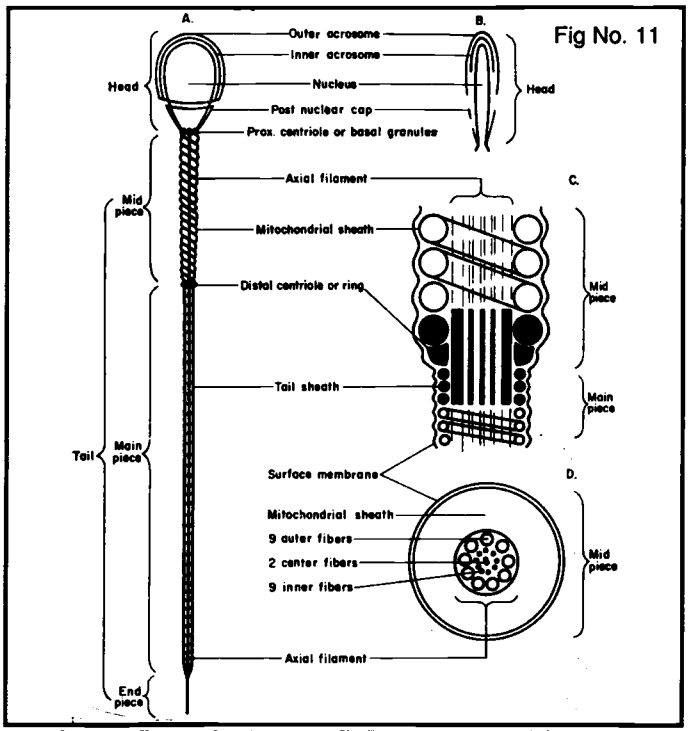


Fig No. 11

Fig. 11 : Diagram illustrating the tentative structure of a typical ungulate sperm. A, General view, (x 2,700 approx.). B, Longitudinal section through the head in a plane at right angles to the paper (x 2,700 approx.). C, Section through the midpiece showing the fibrils and surrounding mitochondrial sheath (x 30,000 approx.). D, Section through the mainpiece showing the fibrils and surrounding tail sheath (x 30,000 approx.). Adapted from Wu, 1966, A.I. Digest, 14, No.6, p.7).

The sperm contains 20% dry matter the largest part of which is the DNA, Protein complex contained in the nucleus. The metabolic processes necessary for providing energy to the sperm take place in mitochondrial helix (Fig. 11).

Table No.1

Size of spermatozoa in domestic animals

Animal	Head		Mid piece		Main piece		Reference
	Length	Width	Length	Width	Length	Width	
Bull (Bos tauras)	9.15	4.25	14.84	0.67	45.50	0.5	Bonadonna et al (1953)
Bull (Bos indicus)	9.22	5.27	-	-	-	-	Tomer et al (1964)
Buffalo Bull	7.40	4.48	12.41	-	43.61	-	Venkatswami and Vandanayagan (1962)
Buffalo Bull	6.62	4.17	9.76	-	42.88	-	Bhosrekar (1975)
Buffalo Bull	8.40	4.92	13.11	-	42.81	-	Sharma (1977)
Camel	5.36	3.42	7.38	-	35.62	-	Khan (1975)
Ram	8.30	4.28	14.0	0.80	45.00	0.50	Marshall
Boar	8.50	4.25	10.0	_	30.00	-	Hancock (1957)

All the figures are in microns

HEAD : The nucleus forms most of the head of spermatozoa. The chromatin consists of 43 per cent of DNA and 57 per cent of arginine rich proteins (Leuchtenberger, 1956).

In spermatozoa the amount of DNA corrosponds to half that of the deploid nuclei. In case of the infertility the amount of DNA does not vary but the quality of basic protein (Histone) alters (Gledhill 1966).

The anterior part of the nucleus is covered by the acrosome. It consists of from outside to inside 1. Cytoplasmic membrane 2. the outer membrane of spermatozoa, 3. the outer memberane of the acrosomal vesicle, 4. the acrosomic material (derived from the acrosomic granule) and the 5. inner membrane of the acrosomic vesicle thickened and pressed against the nuclear membrane. A hereditory anomaly in acrosomic system causes sterility in bulls (Fig. 12).

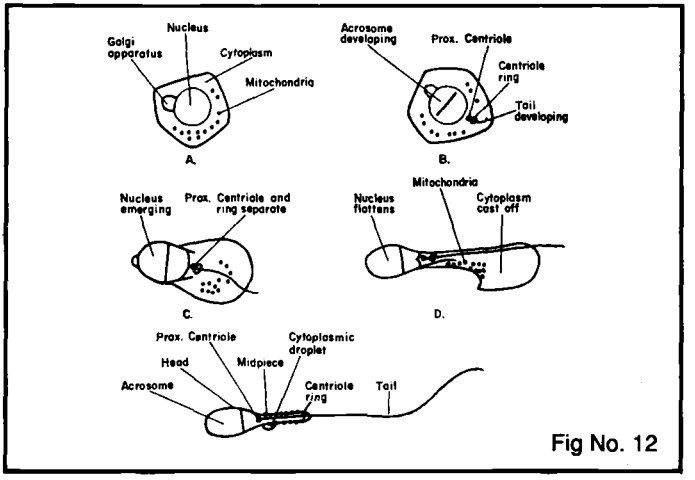


Fig. 12 : Diagram illustrating the transformation of a spermatid into a sperm during spermiogenesis. (From Reproduction in Farm Animals, E.S.E. Hafez ed., Philadelphia, 1962).

The acrosome contains mucopolysaccharides containing galactose, mannose, fucose, and hexasamine (Clermount et al 1955). Acrosome system also contains acid and alkaline phosphatases (Melampy et al 1952). It also contains lipoglycoprotein, which acts as egg envelope and hyaluronidase, Acrosin and other proteinases.

The base of the nucleus is surrounded by the post nuclear cap and also by the cytoplasmic membrane, which is very sensitive to alkali. The post nuclear cap is formed of fibrous proteins rich in sulphur. The post nuclear cap becomes permeable to some dyes such as eosin on death of spermatozoa (Lasley et al 1942).

Neck : The narrow region which connects the sperm head with middle piece, is known as the neck (or neck piece) it is the most vulnerable and fragile part of the spermatozoa.

Midpiece : It is the proximal part of the tail. Due to the arrangement of the fibres there exists only one plane of symmetry, which is perpendicular to the central pair of fibrils. The fibril bundles are enclosed by a spiral of mitochondria.

Tail : The axial filament of the tail is made up of 2 axial fibrils surrounded by two concentric circles of 9 fibrils. The structure of axial filament is of type 9+9+2 derived from the general type 9+2 found is flagellates. (Fig 10)

Main piece : In all spermatozoa of domestic animals the axial bundle of 9+9+2 fibrils contain two longitudinal rings situated on diameter as the central pair of fibrils and surrounded by helix of structural proteins. This whole structure is surrounded by a membrane.

In the caput epididymis the spermatozoa bears a cytoplasmic droplet which migrates towards the distal end of the middle piece from where it is ultimately eliminated on the course of its travel through the epididymis. This droplet contains elements from disintegrating golgi apparatus and endoplasmic reticulum when the droplet is found in the proximal position of middle piece it signifies defect in the maturation of spermatozoa in the epididymis (Lagerlof 1934).

The end piece of spermatozoa consists of only a bundle of axial fibrils covered only by a double layer of cell membrane which also covers the entire spermatozoa.

SPERM MOTILITY

The pacemaker for initiation of sperm motility is located at the base of head "centriole". The rhythmic beats are propogated along the whole length of flagellum towards the tip of the tail. The entire flagellum participates in the whip like movement.

The speed with which spermatozoa moves in a fluid medium is 0.15 mm. per second at body temperature. (Bishop 1962a). The axial filament complex which forms the core of the flagellum consists of number of fine fibrils which run through the entire length of the middle piece and tail. They represent the main contractile element of the sperm cell. The contractions are excited by the rhythmic impulses which occur first in neck and then transported to each fibril in turn. The arrangement in outer bundle of fibrils govern the three dimentional wave like movements. Certain researchers have demonstrated the resemblance of

certain proteins extracted from spermatozoa to those of myosin which is responsible for muscular contraction. Nelson (1958) had shown ATPase activity in nine outer most axial fibrils of rat spermatozoa. There is also chemical evidence that like in muscular activity adenosine triphosphoric acid is involved in motion and metabolism of spermatozoa. Nelson (1962b) has demonstrated by using immunochemical techniques the presence of actin and myosin in axial fibrils.

The progressive motility is promoted by flagellar movements of tail established through contractions of the contractile fibres of the axial filament in a rigid pattern. The sperm head rotates along its axis during progression. Isolated tails are able to move independently. The sperm will move against the stream rheotaxis. The active movement is of importance for transportation of sperm through cervix and for distribution of sperm at the site of fertilization and collision with the ovum; but is otherwise probably of no importance for transportation in the female genital tract. Sperms have been found in the oviducts of females of many species including cattle, sheep within few minutes after mating.

Initiation of motility :It is generally agreed that spermatozoa are immotile or nearly so while in the male reproductive tract although they readily show motility as soon as they are taken out from the tract and examined under the microscope. The quiescence in the tract is because of low partial pressure of oxygen combined with deficiency of glycolysable sugar (Bishop and Mathews, 1952), Walton (1956) reported that inhibition of motility could be produced for several hours by with-holding both sugar and

oxygen. Active motility resumed on supplying either oxygen or fructose, but maximum motility occured only when both oxygen and fructose were supplied together.

Epididymal spermatozoa are active in presence of oxygen only while stallion epididymal spermatozoa are active in presence of glycolysable sugar only (Dutt, 1950).

Salisbury and Cragle (1956), Van Demark and Sharma (1957), Salisbury and Van Demark (1957) and Sorenson and Anderson (1956) obtained evidence that potassium/sodium ratio is high in epididymis as compared to ejaculated semen. Potassium is more in epididymal fluid while sodium is in higher amounts in ejaculated semen. The epididymal sperm gets activated on dilution to normal saline. It is quite clear now that principle activating agent is, increased availability of oxygen and the action of inhibitory agent is reduced in high partial pressure of oxygen. The exogenous source of glycolysable sugar increases the motility and dilution of semen in sodium containing fluid increases its activity.

Factors affecting sperm motility :

Environmental factors influencing the active movements of sperm are, temperature, pH, osmotic pressure and viscosity of the medium. Sperm are reversibly immobilised when cooled to 6°C. Temperature upto 42°C will increase their activity to a maximum while temperature above 46° C will kill the sperm : At pH 6 sperm are immobilised but are revivable even after a short exposure to pH 5. From pH 8 and upwards increasing damage occurs. Both

hypo and hypertonic conditions are toxic to sperm. The former being more hostile. High viscosity inhibits progressive movements (e.g. gelatine in semen diluent).

At ejaculation the immobile sperm become actively motile as they are exposed to 1) sufficient O_2 and a reduced Co_2 partial pressure 2) contact with metabolizable substrate 3) Reduced K and increased Na concentrations 4) Optimal pH, 5) Reduced viscosity and 6) Enzymes and coenzymes necessary for their metabolism.

Energy for flagellar movement is derived from splitting of ATP to ADP + free energy and ADP to AMP + free energy. If the energy rich bonds become exhausted motility will cease. The energy necessary for resynthesis of ADP and ATP is above all derived from enzymatic break down of fructose (fructolysis), under anaerobic conditions leading to an accumulation of the resulting lactic acid, which if sufficient oxygen is present will be broken down to Co_2 and H_2O via kreb's cycle. (Fig. 13 A)

The sperm are able to retain their motility and fertilizing ability in vitro provided the biochemical reactions of release and synthesis of energy are controlled.

Many factors influence these reactions :

Temperature : Sperm are susceptible to sudden cooling (cold shock) which is prevented at semen collection, (collection tube placed in felt cap and before placing it in felt cap the tube is warmed upto 35°C by placing it on warming table), at dilution (the diluent preheated to 35°C is added equal to semen

quantity and allowed to cool down slowly before final dilution with cooled diluent).

pH : Reversible reduction of sperm metabolism by moderate lowering of the pH (Citric acid or bicarbonate and saturation with Co_2 as in the IVT diluent).

Toxic compounds such as disinfectants of all kinds and heavy metal ions and other cell poisons should never get into direct contact with semen. Protection against ultra violet light (direct sunshine) is essential to prevent toxic metabolic products to be produced (H_2 O_2 for example).

Measures adopted to control the motility of sperm in vitro are not necessarily beneficial to the conservation of their fertilizing ability. Depolymerisation of the DNA protein complex is almost impossible to counteract and is probably the cause of the gradual decrease in the fertilizing ability of sperm in diluted semen stored in vitro unless deep frozen.

Distribution of Sperm and Sperm Transport Mechanisms

The reactions and condition of the sperm in the female genital tract are not yet fully understood. At natural mating the bull deposits billions of sperm in the bottom of the vagina and across the external orifice of the cervix. Due to discharge of cervical mucus at oestrus a large proportion of sperm will pass out of the vagina again. However, many millions sperm will penetrate into the cervical canal and make their way through the mucus. This passage is made by the sperm through their own actively progressive movement inspite of the fact that discharge of the

mucus is moving towards vagina. Two factors may help in explaining this phenomenon one is that sperm have rheotactic properties. The cervical mucus, which is composed mainly of mucins at oestrus is roapy and viscoelastic. Due to a stretching of the muco protein molecules forming threads, micelles, get loosely bond together and get arranged in a pattern of many, long canals through which the sperms can pass (Fig. 13)

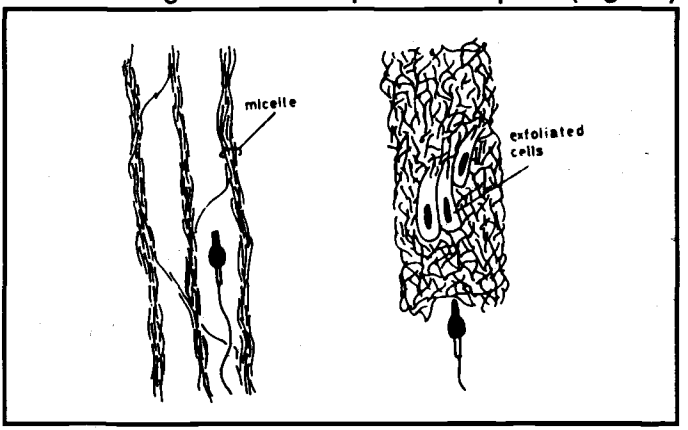
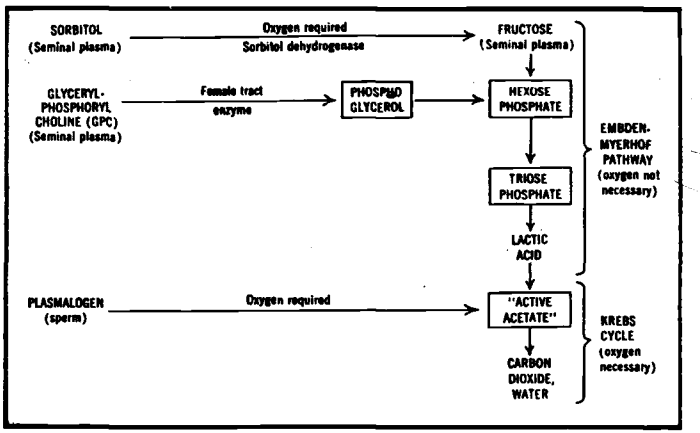


Fig. 13 : Model of cervical mucus showing the ovulatory (oestrogenic) type (left) and luteal type secretion. The oestrogenic model indicates the micelles and intermicellar space open to sperm penetration. The progestational model shows the dense network without micellar structure which acts as a barrier to spermatozoa. (From Odeblad, E.: Acta Obstet. Gynec. Scand. (Suppl. 1) 47 : 59-79, 1968).



(Fig. No. 13A)

Some sperm will move directly through the cervical canal while others will be retained in the crypts in the mucus membrane of the cervix from where they will gradually become released and pass into the uterus. Under conditions of natural mating cervix serves as a reservoir from which sperm are delivered successively in to the uterus through many hours. (Fig 14)

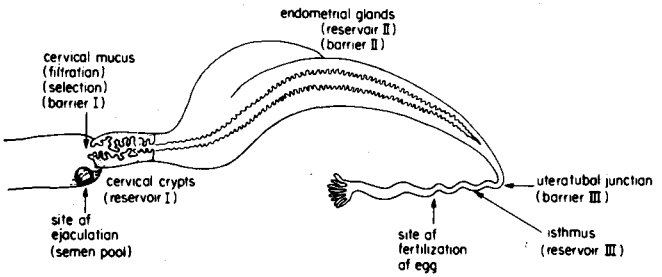


Fig No. 14

Fig. 14 : Diagram showing the various anatomic and physiologic barriers that prevent massive numbers of spermatozoa in the ejaculate from reaching the site of fertilization, presumably to avoid polyspermy.

At A.I. where semen is deposited deeply in cervix or in the internal orifice of cervix or uterine cavity (especially when deep frozen semen is used) the cervix is almost or completely bypassed. The transportation of sperm in the uterus is probably only

mediated by contractions of the uterine musculature. The rhythmic contractions of the myometrium are strongly increased at oestrus and further stimulated by mobilization of oxytocin during the sexual courtship prior to mating or manipulation during the AI process (Rough handling of female must be avoided to prevent reactions provoking an increasing secretion of epinephrine which is antagonistic to oxytocin).

Of the millions of sperm deposited in the cervix and uterus only a few thousand will reach the oviduct and less than a hundred will be present in the viscinity of ovum at the site of fertilization in the ampullar part of the salphinx. (Fig. 15)

Recent studies in sheep (Hunter et al 1980; Hunter and Co-workers 1982) and Pigs (Hunter 1981, 1982) as well as preliminary surgical studies in cattle Hunter and Wilmut, (1983) and Larsson (1988) all clearly show that the functional sperm reservoir i.e. the one drawn upon at the time of ovulation is the caudal portion of the oviduct and not the cervix. Since the ovulation usually occurs 30 hrs. after the onset of oestrus in cattle the spermatozoa are apparently stored in the caudal portion of the oviduct for the period of 16 to 18 hours or more. However, in 1 to 2 hrs. immediately preceeding ovulation there is a descret and active transport of spermatozoa towards the site of fertilization at the isthmic ampulla junction of the oviduct.

Efficient sperm transport in cattle is associated with endocrine controlled changes in the reproductive tract, shortly before and after oestrus. In cattle and sheep the estrogen promotes the secretion of copious amounts of highly fluid cervical mucus and stimulates

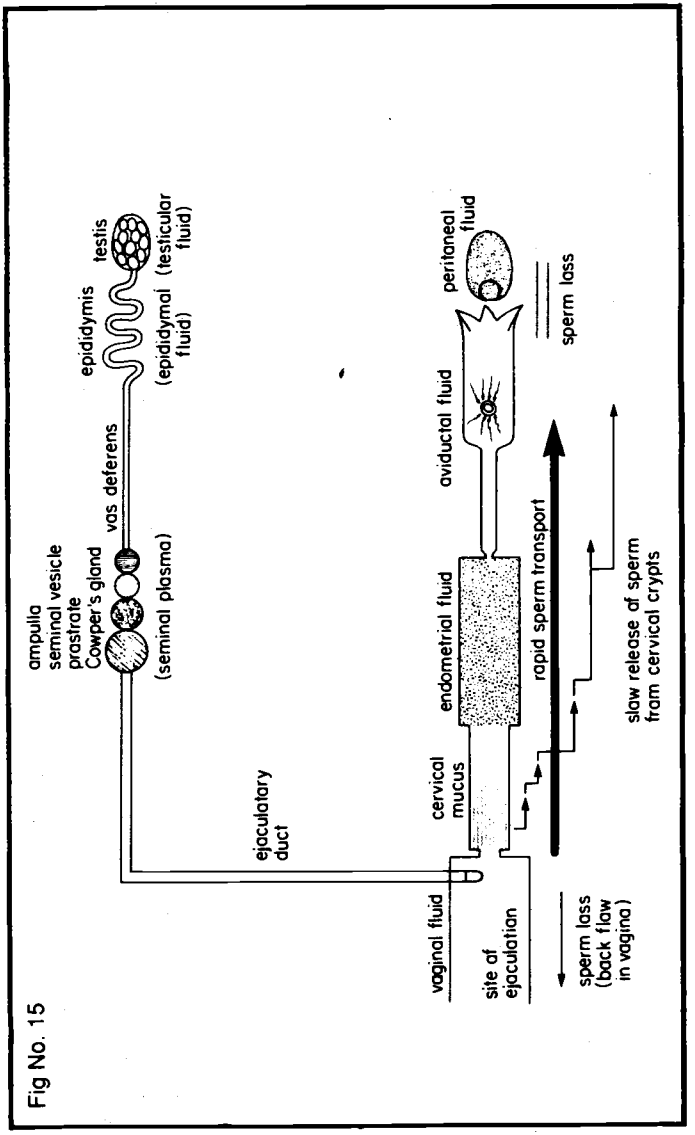


Fig. 15 : Diagrammatic illustration of the various luminal fluids in which spermatozoa are suspended from the time of sperm production in the seminiferous tubules to the time of fertilization in the oviduct.

strong muscular contractions of the reproductive tract. Progesterone, prostaglandins and perhaps other hormones may be involved in the delicately timed movement of sperm within oviduct.

Early in the oestrus the majority of the contractions originate in the cervix or posterior segments of uterus and move anteriorly. Late in the oestrus, the majority of the contractions originate near the uterotubal junction and move posteriorly. The origin of the contractions may be relevant to the sperm transport through out the uterus. It seems therefore, reasonable to postulate that high muscular activity in the posterior segments of uterine horns early in the oestrus would be associated with the transport of sperm through the uterus during prolonged phase of sperm transport and that the increased muscular activity near the uterotubal junction later in the oestrus would be associated with the movement of sperm into the caudal isthmus and perhaps holding sperm in caudal isthmus.

Van Demark and Moeller (1951) recovered sperm from ovarian end of oviducts of cattle as early as 3 or 4 minutes after A.I. or natural mating. Transport of sperms with in minutes to the site has been termed as rapid phase of transport as contrasted to later or prolonged phase. Most of the sperm that pass to oviduct in rapid phase are dead or damaged. Motile sperms adhere to the epithelium or enter the cervical oruterine folds during the phase of rapid transport and avoid rapid propulsion through the tracts. Apparently the sperm transported to the oviducts soon after insemination, even sperm that may be alive are not involved in fertilization.

Capacitation

The sperm are not able to fertilize ova immediately upon their introduction in to the female genital tract, they must first become capacitated. Capacitation is a process involving changes in the acrosomal cap, the outer membrane of which becomes vasiculated and perforations appear through which the contents of enzyme will be released (hyaluronidase, lysosomes, Proteolytic enzymes etc). The action of which paves way for spermatozoa through the follicle cell mass towards the zona pellucida of the egg. The defination of capacitation was updated to reflect all changes upto the morphological aspects associated with "Acrosome reaction" (AR). In that strict sense capacitation is accompanied by a hyper active pattern of motility in sperm. And a flagella may exhibit a whiplash movement. The acrosome reaction (AR) culminates in vesiculation of the plasma and outer acrosomal membranes and release of active hydrolytic enzymes. Presence of calcium is an obligatory requirement for the AR to occur. (Fig 16)

Glycosaminiglycons are unbranched linear polysacharides usually attached to protein core to form a proteoglycon.

Recently glycosaminiglycons have been successfully used to capacitate bull sperm in vitro. Heparin, one class of glycosaminiglycons (GAG) binds sperm in a manner typical of receptor - Ligand interaction. Bulls within above average non return rate produce sperm that exhibit higher frequency of acrosin reaction when exposed to glycosaminoglycons in vitro. In addition the binding affinity, the sperm possesses for heparin is related to non return rates of bulls. The binding

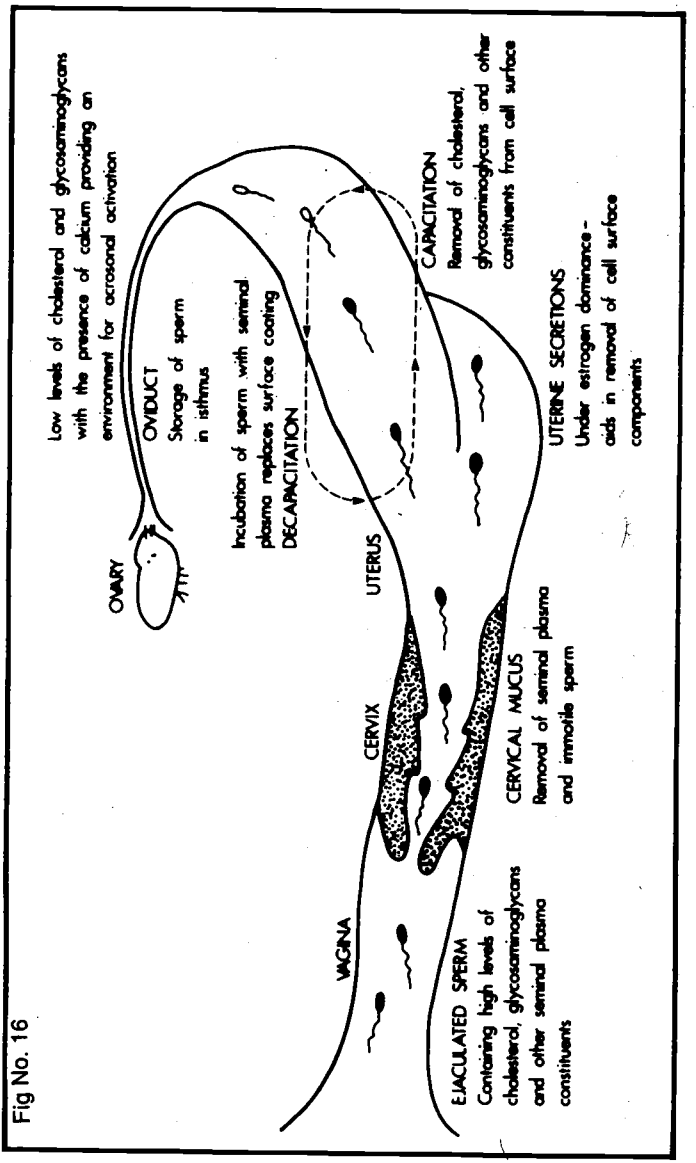


Fig. 16 : Events in sperm capacitation during transport through the female genital tract. (Drawn by Dr.Geisert)

affinity decreases in semen samples with high frequency of abnormal sperm. Glycosaminiglycons may be useful probes to evaluate in vitro cellular changes that sperm completes prior to fertilization and serve as markers of sperm membrane defects which ultimately affect fertility of bulls.

Glycosaminiglycons (GAG) do not directly lead to AR but rather they predispose sperm to respond to calcium and trigger membrane changes typical to AR.

In vitro Heparin behaves like GAG. Promotes uptake of Ca ++ in bull sperm. Ca ++ is necessary for the membrane alternations characteristic to AR.

Glycosaminiglycons have been identified in female reproductive tract from several mammalian species. Estrual secretions from cow were more effective at inducing AR in bull sperm than the samples collected at diestrous. The ability of estrual secretions to capacitate bull sperm presumably is due to secretion of more heparin like material under estrogen dominance.

The other sources of GAG that bull sperm could encounter in the cow are hyaluronic acid secreted by cumulus cells surrounding the oocyte and dermaten sulphate and heparin sulphate from follicular fluid. The zona pellucida also contains GAG like material. The presence of GAG from various sources in the female tract would provide for a continuous selection process of sperm traversing the female reproductive tract and undergoing capacitation. The sperm that ultimately fertilises the egg probably completes its final cellular changes at the time of fertilization but other sperms are going through a natural selection

over a time and are being removed from the population that could potentially fertilize the egg. (AX and Lenz (1987).

Fate of deposited sperm :

Fertilization reflects the success of sperm transport and fertilization failure probably reflects the failure of sperm transport. A gradient was apparent in sperm numbers through the reproductive tract with numbers decreasing from several millions in the cervix to several thousands in the oviducts. In an experiment 2 billion sperm were deposited in the external 'OS' of Hereford heifers and heifers were necrospid at 1 hr. 8 or 24th hour after AI. The entire reproductive tracts was separated in to various segments and sperm were counted in the flushings of each segment. Not only the sperm number decreased drastically from vagina to the oviducts but a rapid and sustained decline was also apparent in the total number of sperm in the entire tract. Of the total number of sperm deposited 13.4% recovered at 1 hr, 3.8% at 8 hrs. and 0.9% at 24 hours. (Table 2)

Most of the missing sperm are phagocytized. Phagocytized sperm are seen in all parts. Sperm have been found in reproductive tract of cow till 72 hours after A.I.

The de-capacitation factor is present in seminal plasma, which can be separated from seminal plasma by ultra centrifugation at 105000 g. for 3 hours (Bedford and Chang 1962 a). The uterine fluid contains the capacitation factor in abundance in post ovulatory stage.

Table No.2

Spermatozoa recovered from reproductive tract of Hereford heifers after AI

Time after AI (Hrs.)	Total Number of Sperm recovered 10^6	Proportion of inseminate recovered %	Number of sperm recovered				
			Vagina (10^6)	Cervix (10^6)	Uterus (10^6)	UT (10^6)	Ovi duct (10^6)
1	269	13.4	207	59	2.9	40	24
8	76	03.8	51	20	5.3	150	200
24	18	00.9	10	5	2.7	60	15

Insemination per heifer was 2 billion sperm

CHAPTER III

COMPOSITION OF SPERMATOZOA AND SEMINAL PLASMA

Spermatozoa

The spermatozoa possesses two highly specialised functions motility and fertilizing capacity, which are located in two different parts of sperm structure. The sperm head which incorporates the nucleus carries deoxyribonucleoprotein, which is the most important constituents in so far as the fertilizing capacity of spermatozoa is concerned. The sperm flagellum which comprises the midpiece and tail is the organ of motility and carries all the enzymes and conenzymes needed for metabolic activity.

Sperm DNA when separated from nuclear protein is composed chiefly of four nucleotides each consisting of one molecule of phosphoric acid, one molecule of sugar, deoxyribose and one molecule of purine or pyrimidine base. (Adenine, guanine, cytosine or thymine). With in species all spermatozoa carry constant amount of DNA, while RNA is virtually absent from mature spermatozoa (Mann 1951). The protein conjugated with DNA are of basic type and have been shown to be either protamines or histones. In addition to DNA the sperm head carries several other important constituents including some carbohydrates in acrosome. A lypoglycoprotein, composed of phospholipid and glycoprotein, several amino acids and carbohydrate components are present. The chief among these may be glutamic acid, aspartic acid, leucine, alanine, serine, glycine and proline and

mannoses, galactose, fucose, glycosamine, galactosamine and sialic acid. The whole lipoglycoprotein complex as prepared from ram and bull spermatozoa exhibit characteristic proteolytic and hyaluronidase activity and is capable of dispersing cumulus oophorous and carona radiata (Srivastava et al 1965).

In addition the sperm head contains several other residual proteins with in the category of soluble non basic nuclear protein containing tryptophane and another much less soluble sulphur rich, keratin like protein, which is believed to constitute the sperm membrane.

The middle piece and tail together constitute, the sperm flagellum. The mitochondrial sheath is rich in phospholipid, most of it is bound to protein, some phospholipid is also present in other part of spermatozoa which appears as choline plasmologen.

Other important components of the mitochondrial sheath are the complete cytochrome system and a number of enzymes viz. succinic, malic, isocitric, 6 phosphoglucose and lactic enzymes, oxydizing enzymes like sorbitol dehydrogenase,lactic dehydrogenase, ATPase etc.

Seminal Plasma

The extra cellular fluid, which provides the medium vehicle for spermatozoa is a composite mixture of secretions, which comes from the male accessory organs, of reproduction. The seminal plasma in higher mammals is proportionately more than the mass of spermatozoa. It is the secretion from epididymis,

Table 3

SEMEN CHARACTERISTICS OF DOMESTIC ANIMALS

Semen Constituents	Buffalo	(FROM ROBERTS 1971) Bull	Stallion	Ram/Goat	Boar
Volume (ml)	2.5 (2-6)	4.0 (1-15)	70.0 (30 to 250)	1.0 (0.7 to 3.0)	250.0 (125 to 250)
Spermatozoan concentration millions/ml.	800 (300 to 2200)	1200 (300 to 2500)	120 (30 to 600)	3000 (1000 to 6000)	150 (25 to 1000)
pH	6.8 (6.6 to 7.0)	6.6 (6.2 to 7.5)	7.4 (7.0 to 7.8)	6.6 (6.2 to 7.0)	7.4 (7.0 to 7.6)
Fructose Mg/100 ml.	450 (300 to 900)	530 (150 to 900)	2 (2 to 6)	250 -	13 (3 to 50)
Fructolytic index	1.44 ± 0.11	1.99 ± 0.15			
Glyceryl phosphoryl choline Mg/100 mol.	-	350 (100 to 500)	- (40 to 120)	1650 (1100 to 2100)	- (110 to 240)
Potassium (mg/100 ml)	-	140 (80 to 210)	60	90 (50 to 140)	240 (80 to 380)
Sodium (mg)/100 ml		230 (140 to 280)	70	190 (120 to 250)	650 (290 to 850)
Inorganic Phosphorus	6.40 ± 0.60	5.90 + 0.50			
Phosphorus total (mg)	103.20 ± 8.90	80	17	375	66
Organic phosphorus	68	59.90			
Citric acid mg/100 ml.	489	700 (300 to 1100)	25 (8 to 60)	140 (110 to 260)	80 (60 to 100)
Inositol mg/100 ml.	-	35 (25 to 46)	30 (20 to 47)	12 (7 to 14)	530 (380 to 630)
Sorbitol (mg/100 ml.)	-	(10 to 140)	40 (20 to 40)	92 (76 to 120)	12 (6 to 16)
Ergotheonine	-	0	(40 to 110)	0	(6 to 23)
Ca. Mag	40.50 ± 2.10 -	44 (35.60) 9 (7-12)	20 3	11 (6-15) 8 (2-13)	5 (2-6) 11 (5-14)
Chloride	369 -	180 (110-290)	270 (90 to 450)	86 -	330 (260 - 430)
Protein g / 100 ml.	-	6.6	1.0	5.0	3.7

All the figures except otherwise mentioned are in mg / 100ml.

vasdeferens, ampullae, seminal vesicles, prostate, cowper's glands and urethral glands. (Table 3)

Composition of seminal plasma

The average specific gravity of seminal plasma is 1.03 that of sperm is 1.075 while semen has 1.035 dependent on sperm concentration.

Osmotic pressure of the seminal plasma corrosponds to that of blood plasma i.e. freezing point depression $\Delta = 0.53^{o}C$. Freshly collected semen has a pH of 6.4 to 6.8 dependent upon the relative amount of partial secretions. Lower values are seen upon standing at room temperature (production of lactic acid under anaerobic metabolism). Higher pH values are found in semen having got contaminated with bacteria, (NH3 formation from deamination processes) in successively collected ejaculates, incomplete ejaculates (in bulls inadequately prepared, faulty collection technique), and in inflammatory conditions of testis-epididymis.

The seminal plasma has a poor buffering capacity. The composition of bovine seminal plasma is very complex and can be divided into 1) Inorganic substances 3) Nitrogenous bases 4) Proteins, enzymes, amino acids 5) Vitamins 6) Organic acids and 7) Lipids.

Inorganic Substances :

Sodium, potassium, calcium, magnesium, chloride, Co_2, (hydrogen carbonate) phosphorus and sulphur mostly bond in organic compounds and Iron, copper, zinc mostly bond to enzymes.Seminal plasma has a

higher sodium than potassium while the opposite is true for spermatozoa. Exchange of Na + and K + between sperm and seminal plasma is an active transport across the cell membrane and is of importance for contractile mechanism of the sperm tail fibrils.

Carbohydrate :

Fructose is the major carbohydrate in bull semen (average 0.5%) and is formed from blood glucose. Two more polyols viz. inositol and sorbitol are found. The latter can be oxidised to fructose when sufficient oxygen is present inositol is possibly helping in maintaining the osmatic pressure.

Nitrogenous Bases :

Glycerophosphoryl choline is the most important and (0.4 to 0.5%), is not metabolised by sperm, but can be split by the enzyme present in the female genital tract (diesterase) into choline and phosphoglycerol. The latter is metabolizable by sperm.

Protein, amino acids etc :

90% of N in seminal plasma is bound to protein. Only minute amounts are present as NH3, uric acid and urea. Total protein contents in bovine semen is 7% mainly as globulins. 18 amino acids have been demonstrated. Most of them in their free form. Glutamic acid constitutes more than 50%.

Enzymes in semen are mainly bound to sperm. Those found in seminal plasma have leaked out from damaged or dead sperms. Proteolytic enzymes (e.g.

Trypsin like) phospholipases, Transaminases e.g. Glutamic oxaloacetic transaminase (GOT used as test for sperm membrane damage) glycosidases (e.g. ß. glucoronidase) a complete set of cytochromes, hexokinases and dehydrogenases (necessary for metabolizing fructose), a number of phosphatases (Acid and Alkaline). ATPase (Through splitting of adenosin triphosphate furnish direct energy for sperm movement) 5 nucleotidases (splits ribose 5 phosphates) DNA ase (Possible functioning by splitting DNA in dead/degenerating sperm in vivo) and hyaluronidase (active at sperm penetration into the ovum by splitting hyaluronic acid) are examples, Catalase is not present in sperm or seminal plasma (used as a test for contamination with pus and bacteria).

Mucoproteins (e.g. sialo-mucoprotein as such or split off sialic acid are of importance for adhesion of sperm to ovum at fertilization.

Vitamins :

Only water soluble vitamins are found in semen. Vit. B complex (riboflavin, the major component responsible for yellow semen). Ascorbic acid (partly responsible for reducing properties).

Organic acids :

Most important is lactic acid (as intermediary step in the anaerobic fructose break down). Acetate, Pyruvate, citrate succinate (the two former metabolizable by sperm) Prostaglandins (in some species possibly of importance for semen transport in

female genital tract through their effect on myometrial contractions) are also present in bull semen.

Lipids :

Mainly phospholipids (above all plasmalogen and some lecithin) and small amounts of cholesterol, di and triglycerides. Bull semen also contains androgens (Testesterone, dehydro androsterone) and minute amounts of estrogenic hormones.

The composition of seminal plasma is dependent on testosterone. Fructose and citric acid do not appear until puberty and disappear after castration but will reappear in castrated males after administration of testesterone. (Fructose test and citric acid test have been utilized for studying androgen production by leydig cells.)

The Accessory Glands

The main accessory sex glands in the bull are ampullae, the seminal vesicles, the prostate and cowpers glands (also known as bulls urethral gland) (Fig.10) all of which produce secretions which contribute to ejaculated semen. There are also other urethral glands and preputial glands.

The ampullae appear as dilations of the urethral end of the ductus deferens. They are well developed in the stallion and absent in the boar. The vasa deferens transport semen from the tail of the epididymis to the urethra. The outer diameter is 2 mm and its thick muscular wall makes it feel firm and cord like. The two different ducts lying side by side above the bladder

gradually become thicker in diameter thus forming the ampullae.

This thickening of the duct is due to abundent occurance of the glands in the wall. The ampullary glands are tubular and histologically very similar to the structure of vesicular glands. The two ampullae pass under the body of the prostate and open together with the excretory ducts of vesicular glands in to the urethra. During courtship and precoital stimulation (teasing) the sperm are transported from the tail of the epididymis to the ampulla aided by the peristaltic movements of the vasdeferens.

The ampullar glands secret small amounts of fructose and citric acid.

Vesicular glands :

They are paired, markedly lobulated glands situated in the urogenital fold lateral to the ampullae. The vesicular glands may vary considerably in size and lobulations between individuals. The secretory ducts from the individual lobules form one main excretary duct which is located in the centre of the gland and extends posteriorly under the body of the prostate. Each excretory duct unites with the different duct at its outlet into the urethra. The secretion of the gland is faintly opalescent, sticky fluid. It contains a high concentrations of proteins, potassium, citric acid, fructose. It is often distinctly yellow in colour because of high flavin content. The pH varies from 5.7 to 6.2. The secretion of vesicular gland constitutes 60% or more of the volume of normal ejaculate in the bull. It also contains ascorbic acid, ergothionine, inorganic

and soluble phosphorus, sodium, ATP and several enzymes.

Prostate :

The prostate gland which surrounds the urethra is composed of two parts : Body of prostate (corpus prostate) and the disseminate or cryptic prostate. The secretions of these two parts pass through the numerous small ducts which open into the urethra in rows.

The prostate gland secretes citric acid, fructose ergothionine, acid phosphatase. It is a source of calcium, sodium, potassium chloride and bicarbonate. It is rather high in Zinc (9.4 to 11.3 mg/100 g of fresh tissue). The protein content is very low. The secretion is acidic pH 6.4.

Cowpers glands :

It is a paired glands. They are round compact bodies with a dense capsule and are about a size of walnut in the bull. They are located above the urethra near its exit from the pelvic cavity. The secretary ducts from each gland unite in one excretary duct which is 2 to 3 cm in length. The two excretary ducts of the gland have small separate openings in the margin of the mucosal fold of urethra.

Both prostate and cowpers glands are lobulated, tubular glands and the thick septa between the lobules contain unstriated muscle as in the vesicular glands. These muscles enable the glands to discharge their secretion suddenly. The secretion is viscous rubber like white. Secretion is distinguished by a high content

of a sialo protein. The viscous fluid plays an important role in formation of "gelation" which occurs in Boar seminal plasma at the time of ejaculation and immediately afterwards. As the gel is formed the sialo protein swells and is incorporated in it leaving behind the liquid portion of the semen. The pH of the secretion is alkaline 7.0. The low protein and high chloride content of the secretion is rather hostile to sperm. The dribblings from the prepuce of the bull before mounting are secretions from cowpers' gland. Most probably their function is to flush urethra.

Epididymis :

In addition to its repository function for spermatozoa it also produces secretion and participates in production of seminal plasma complete absence of fructose or any other glycolysable substance is one of the interesting feature of this secretion. Another interesting feature is its high potassium low sodium ratio. Salisbury and Cragle (1956) recorded 373 K/14 Na in head, 177 K/90 Na in tail of epididymis. 240 K/ 160 Na in ampullae and 287 K/250 Na mg/100 ml in vesicular glands. Another interesting feature of epididymal gland is its high content of Glycerophosphoryl choline. Amongst enzymes there are several active glycosidases including ß glucoronidases and mannosidases, ß galactosidases, ß & α fucosidases and ß N acetyl glucosaminidase. Some of these enzymes especially mannosidases and ß N acetyl glucosamidase occur in extra ordinary concentrations in epididymal seminal plasma.

Urethral glands

The secretion of urethral gland is rich in mucoprotein. It is clear and watery. The pre sperm fraction which is

emitted at the time of ejaculation prior to spermatozoa is essentially urethral gland fluid.

Preputial glands :

Preputial gland is rich source of ß glucoronidase. It also produces 7 dehydro cholesterol (Ward and Moore, 1955).

The function of all these glands in dependent on the secretion of the testosterone by the testis.

Physiological significance of seminal plasma and accessory secretions

1. It is a natural diluent for spermatozoa. Spermatozoa could scarcely be expected to function without the provision of seminal plasma.

2. It is vehicle for spermatozoa. It is the best medium since it is well buffered and contains energy source for spermatozoa.

3. It exerts distinct stimulatory effect on spermatozoal motility. This is simply due to "dilution effect".

4. It contains ready source of energy for spermatozoa.

Prostaglandin and vesiglandin

Quite apart from the general effects on the animal as a whole male accessary secretions are capable of stimulating isolated smooth muscle organs, such as strips of uterus or intestines.

The pharmacodynamic effects of human seminal plasma received a powerful stimulus. The work of Von Euler in Sweden (Von Euler 1934 and 1949) and Gold batt in England (1933 to 1935) culminated in the discovery of prostaglandin and vesiglandin.

Goldbatt discovered that the oxytocic activity of human seminal plasma can be destroyed by short boiling of seminal plasma with 0.1 N NaOH or 0.1 N HCL. Von Eular (1936) as a result of his experiments on the oxytocic properties of human seminal plasma came to the conclusion that the uterus stimulating activity though possibly be due to several substances can be largely accounted for by Prostaglandin a specific seminal constituent capable of stimulating the contractions of strip of human uterus as well as the isolated strips of uterine tissue from various other animals such as the cow, rabbit, guinea pig and rat. The observations were later confirmed in experiments with purified preparations of prostaglandin. Prostaglandins were thought to be of prostatic origin but now it is regarded as a product of seminal vesicles in man and ram at any rate (Eliasson 1959). This is a mixture of closely related unsaturated fatty acids.

Prostaglandins are of two types E and F, and both of them are active smooth muscle stimulant. In addition to this prostaglandin E has a depressing effect on blood pressure.

Factors affecting semen production

Age : The age of first semen donation varies from breed to breed. The bull calves of exotic breed donate semen as early as 12 months, while bulls of tropical breeds donate at much later age, Tharparkar 36

months, Red Sindhi 34 months, Sahiwal 36 months, Hariana 40 months, Murrah 40 months, Cross-breed 26 months (Bhosrekar, 1988). As the age advances the quantity of semen also improves. Upto 6 years good quality and quantity is expected from bull. Above 6 years there is a decline in quality of semen in tropical breeds. In exotic breeds optimum quality of semen can be obtained upto 62 months in Jersey and 50 months in Holstein Fresian, (Bhosreker, et al1983).

Significant increase with increasing age was found in sperm concentration, sperm motility, and total sperm output, Scrotal circumference and width increased by 32% from puberty (41+1 week) to 2 years of age (Almquist et al 1976). The authors are of the opinion that Scrotal circumference could not be precisely used to predict the sperm output. Sangaev (1977) was also of the opinion that semen quality as well as quantity is affected by age. Earlier Bratton et al (1956) Van Demark (1956) also reported significant effect of age of semen characteristics.

Body size :

Not only age but body size also plays very important part in influencing semen production. Well built bull with higher body weight is expected to donate higher quantity of semen as compared to short statured and with lesser body weight.

A strong positive correlation between body weight and testis size was observed by number of research workers (Coulter and Foote 1977); Kupferschmied et al 1975; Huestan, et al 1988). Bhosrekar et al (1980) showed high positive significant correlation within body weight and scrotal volume in HF and Jersey

bulls. Maximum scrotal volume was obtained at 37 months in HF at a body weight of 470 kg and 36 months in Jersey at 370 kg body weight, while Salam Daudu and Shoyinka (1983) have shown positive correlation between Testicular size, testicular weight and sperm production. Szerahalyl (1988) has shown that body weight bears a significant correlation with sperm concentration and sperm motility in Holstein Fresian and Simmentals. Similar observations were recorded by Jakubiee (1984) for polish black and white low land bulls. Bhosrekar et al (1988) have observed effect of body weight as well as age in the production of semen. As the age advanced the quality in terms of sperm concentration and initial motility improved in Holstein Friesian, Jersey as well as cross-bred and buffalo bulls at Uruli-Kanchan.

Breed/species :

Breed differences were recorded for semen production Warnick et al (1969) recorded breed differences in testicular size and semen characteristics. They examined Angus, Brahman, Hereford, Santa Gertrudies breed. They found significant breed differences. They found overall highest semen quality in Hereford. Verma (1973) studied Ayrshire Jersey and Murrah buffalo bulls. He found significant differences between breeds for ejaculation volume. Barbulescu et al (1965) studied Brown swiss Romanian spotted and Estonian Red bulls and found significant breed differences in ejaculate volume. Bhosrekar et al (1980) did not record any differences on account of breed for ejaculate volume, sperm concentration and number of doses frozen for Holstein Friesian and Jersey breeds. However, they recorded significant differences for cross-bred and buffalo bulls.

Kulkarni and Bhosrekar (1973) also recorded no breed differences for ejaculate volume and sperm concentration between Brown Swiss and Tharparkar, however, they recorded significant lower reaction time in Brown Swiss as compared to Tharparkar.

Nutrition :

It is generally felt that a good all round balanced diet is essential for optimal sperm production. It should not be too fatty diet and should be well balanced. Under nutrition or low plane of nutrition delays maturity, sperm concentration goes down. The quantity of semen also is reduced. Too much concentrate ration also causes irritation of prepuce because of small pustular growth, and bulls do frequent masturbation. Too bulky diet also should be avoided. Greens, concentrate and minerals as well as vitamins in right proportion will keep the bulls trim and healthy.

Numerous studies showed that nutrients of animal origin are superior to that of vegetable origin for sperm production in several species including boar, ram and bull. However, Prabhu et al (1953) and Flipse et al (1956) failed to support this contention.

Bulls grow faster to a adult size, hence their requirement for concentrate and forages is higher as compared to heifers. It has been observed that when energy intake is restricted growth rate is decreased and age of puberty is increased; growth of testis is retarded and sperm out put is reduced. James (1950) using 5 pairs of identical twin bulls fed on two different nutritional levels from 20 weeks to 2 years of age found that at 15 to 24 months of age the bulls in the low plane group had a reduced level of total sperm

production and smaller testis than those in higher plane group. Devies et al (1957) using one set of identical twin calves reported reduced sperm production and retarded onset of spermatogenesis in the calf on low plane of nutrition. Bratton et al (1956) found no significant differences in conception rates among bulls fed on high, normal and subnormal nutritional levels.

Several studies have been reported on the effect of feed level and type of ration on semen production, libido and fertility of adult bulls, Mann and Walton (1953), Prabhu et al (1953) recorded no effect on semen characteristics. Recently Bhosrekar et al (1988 a & b) also found no effect by increasing or decreasing 20% DCP or energy in ration of adult cross-breed bulls on their semen quality and quantity. Bhosrekar et al (1988) also fed adult cross-bred bulls on bagasse based complete diet with only 5 kg green fodder for 15 months and found no effect on the semen production or quality.

Physiological factors :

The relationship between the gonadotrophins from anterior pituitary (FSH and LH) and testis is not cyclical as with the ovaries and therefore, considerably simpler. The action of FSH without contamination with LH is still uncertain. It would seem that on its own it stimulates development of spermatic tubules and does not stimulate either development or secretion of leydig cells of testis. The presence of LH is known to affect both the qualitative and quantitative results of administration of FSH. In the mature hypophysectomised male, FSH administration maintained the weight of testis. Its continuous

administration caused the development of spermatozoa but the interstitial cells were not fully maintained and accessory sex glands were also of the same size as untreated controls, indicating no secretion of androgens. LH is therefore, thought to be responsible for production of androgens from testis.

No increase in the sperm production was demonstrated by injection of gonadotrophins. The maintenance of accessory reproductive tract depends on an adequate secretion of androgen by the testis. In mammals this hormone is Testosterone, castration is followed by failure of development of the secondary sexual characteristics followed by loss of libido and assumption of more female appearance. Since androgens control the development of secondary sex glands, they reduce their secretions on castration, penis gets reduced in size as sequele to castration atrophy.

In normal male, the injection of androgens or of gonadotrophins usually has little effect on sex drive, but these substances have been used to promote sex interest in young males. P.M.S. gonadotrophins (which resembles FSH in action) seems useful and less likely to damage the animal gonads through suppression of pituitary FSH output than an androgen.

Estrogens have no effect on intact male but castrated males show growth of mammary gland. The intact male is protected by its own endogenous androgen production. The effect of plant estrogens on grazing with early subterranian clover (Bennetts et al, 1946) is more in female and withers than in entire males.

Genetical Factors :

Hypoplasia of testis is caused by single autosomal recessive gene. It is described by Lagerlof (1934). This may be one sided or double sided. It may be partial or total. In total hypoplasia there is absence of spermatogenesis the seminiferous tubules show no germinal epithelium. In partial hypoplasia some seminiferous tubules show spermatogenesis. The sperm concentration is very poor and inconsistant and causes subfertility or infertility in bulls. Segmental aplasia of the wolffian duct causing absence of some part of reproductive tract affects the sperm production.. Congenital sperm abnormalities (sticky chromosomes, dag defect and other head abnormalities) are also hereditary in nature (Bane, 1954; Knudson, 1954) Spermeostasis, torsion of testis, upward diversion of penis all are hereditary in nature and influence sperm production and cause infertility amongst bulls.

Seasonal variation :

Seasonal variation in ejaculate volume mass activity, initial motility, sperm concentration. M.B.R. time, live count has been shown by number of workers in India and abroad, especially tropical breeds are affected because of photoperiodicity and ambient temperature. Buffaloes are most vulnerable to these changes. (Prabhu and Bhattacharya, 1951; Oloufa and Coworkers, 1959, EL Sawaf, 1971; Shalash 1972; Kodagali, 1972; Porwal et al 1972; Bhosrekar 1975). They also showed seasonal variation in semen production in buffalo bulls, winter season being more effective in causing deteriorating changes in sperm concentration, live count, MBR time motility etc. If optimum feeding regime is maintained and bulls are

protected against climatic odds seasonal variation in semen production can be easily controlled. Male goats (Bucks) also show great influence of season on donation of semen, higher climatic temperature of summer reduces the libido in bucks and buffaloes and they refuse to donate semen if taken late in the day after 9 a.m. But if the goats and buffaloes are washed before and collection is taken early in the day before 8 a.m. they readily donate even in summer. Cattle bulls of exotic breeds also show similar influence of season on semen donation and its quality. Exotic breeds loose their libido in excessive high ambient temperature but if kept under comfortable conditions with fan and sprinkling of water semen donation is not affected. Only sperm concentration goes down and more watery semen is donated.

Season significantly affects ejaculate volume in bulls, buffalo bulls as well as bucks. Summer is the worst causing lowered ejaculate volume while winter is the best giving highest ejaculate volume. With the onset of monsoon ejaculate volume improves in buffalo bulls (Ibrahim et al 1983, Bhavsar, et al 1986, Bhosrekar et al 1988). Seasons influenced the cross-bred bulls. The semen parameters in cross-bred bulls were significantly affected by seasons. Season of winter (Nov. to Feb.) is found better as compared to other seasons for semen production in cross-bred bulls, (Bhosrekar et al 1986), Bhosrekar et al (1985) reported that ejaculate volume, mass motility and total sperm abnormalities are significantly affected by seasons in cross-bred bulls. Summer proved to be detrimental to sperm morphology, ejaculate volume and mass motility.

Holy (1971) in Holstein Friesian bulls in Cuba, Kalev et al (1968) in Red Dane bulls, Igboeli and Rakha (1971) in Angoni bulls in Egypt, Rao and Rao (1975) in Jersey bulls and Bhosrekar et al (1980) in Holstein Friesian and Jersey bulls reported similar findings. Belorkar (1986) reported marked seasonal variation in sperm concentration, live count and sperm abnormalities in cross-bred bull's semen.

Psychic factors :

Bonadonna (1956) has stated that physiological ejaculation of semen is a complicated phenomenon which can be regarded as completion of a coordinated sequence of responses, some of which are congenital or unconditioned and others acquired or conditioned.

This starts with the sexual excitement and reaches a climax at the so called ejaculatory thrust, typical of ruminants. The jumping impulse is among the congenital reflexes and are peculiar to species and are readily subject to alteration. Whereas conditioned reflexes depend on training and rearing methods. Any psychic trouble, even if irrelevant from a clinical point of view, may seriously affect the sexual function of a bull. Conditions such as traumatic reticulitis or the onset of tuberculosis may be cited as some examples. The most important acquired responses are inhibitory as they may break the performance of copulation or render the sequence of events leading upto ejaculation less efficient and cause production of semen of impaired quality. Such inhibitory responses to fear, pain or other unsuitable conditions are readily developed and very hard to eliminate.

Apart from such acquired responses certain unusual events at the time of copulation or semen collection such as the visit of stranger to a bull centre, can cause the production of poor ejaculates or even deterioration of semen. This difficulty can be over come by training bulls to ignore other destracting surroundings, but other factors like floor of collection shed, roof of collection shed, attendants, dress of attendants, dummy or stimulus animal, frequency of collection interval between two ejaculates, temperature of artificial vagina, the collector of semen, his behavior towards bull can cause variation in semen donation and quality of semen. For example too low roof develops fear in the mind of bulls of getting colloided on mounting the stimulus animal, so he hesitates in mounting. Cement concrete floor causes slipping of hooves of bulls on mounting which causes variation in semen quality and quantity. The bulls should get sufficient grip on the ground at the time of thrust. Undulating matting or worn out matting causes dismount difficulty and the bull falls which carries disappointment and next time he hesitates mounting.

Similarly short or tall dummy for bigger or short bulls respectively create difficulty in mounting. More attention should be given to the selection of a teaser, since a bad animal may repel the bull rather than attract or sexually stimulate him. Teaser should stand quietly in the crate, should not be kicking or jumping.

Teaser should not have wide pelvis as the young male can not hold on to the dummy at the time of mating. (Milk Marketing Board report 1954)Even the change in timing of collection upsets the bull. Buffalo bulls are very temperamental in semen donation. If the bull is beaten or hit by other bull the question of

semen donation on that day does not arise, on the contrary the bull will never donate semen. The fear is highly inhibiting factor. For normal collection from buffalo bull the stimulus animal or teaser should be preferably buffalo cow (Bhosrekar, 1975).

If a dummy is a cow, the bulls readily donate semen but if the dummy is cow in heat, the bulls not only donate semen quickly but of an excellent quality (Prabhu and Bhattacharya 1954). Experience with rubber matting along with coir matting for covering the floor is good. This type of matting helps in jumping. Dust can be avoided. Some of the bulls do not donate semen in the roofed enclosure they require open space and pucca ground. Some bulls do not donate semen on one dummy all the time. They find monotony and require a change in dummy. As soon as dummy is changed the bulls donate semen by giving strong ejaculatory thrust.

Buffalo bulls require longer time for training as compared to exotic bulls. Similarly indigenous bulls also take longer time to get trained for donation of semen in Artificial Vagina. Buffalo bulls can be trained to donate semen in AV on male buffalo bull without much problem if they come from the same herd. If the buffalo bulls live for longer time in one herd or station they do not find any fear from one another and donate semen in AV when collected on another male buffalo bull. On the countrary if the buffalo bulls are coming from different herds they trouble for training. Those adult buffalo bulls used for Natural Service can be trained to donate semen in AV provided female teaser in used. Exotic and cross-bred bulls do not pose any problem in training.

Pressure stimuli :

Site of collection, sight of stimulus animal, attendant and the semen collector can be the source of stimuli for onset of reflex for mount and ejaculation. The whistling sound or a pat on dummy also create reflexes for collection. Smelling power of the bull seems to be very potent stimulus to the bull for mount and ejaculation. In order to maintain optimum pressure stimuli the site of collection, the dummy animal, the attendants and the collector should not be changed. Any change in normal set up will increase the reaction time and reduce the quality as well as quantity of semen (Bhosrekar et al, 1988). Changes in set routine, e.g. schedule of collection, interval between collections (days) and ejaculates, feeding regime, and other managemental practices like washing of bulls etc., do create problems in obtaining good quality ejaculates from bulls.

Bhosrekar et al (1986 a and b) found that morning time for collection is most suitable as compared to evening hours, as morning time is pleasant, less destracting and fresh. In the afternoon the bulls are full stomached, there is lot of destraction of attention of bulls because of the visitors, evening ambient temperatures are higher as compared to morning. It has been observed both in exotic as well as cross-bred bulls the semen parameters viz. ejaculate volume, sperm concentration are higher in morning collection as compared to evening, even the freezability of semen is better for morning collection.

Variations should often be introduced in preparation and restraint of bulls e.g. Quick two false mounts and collection some times prove better as regards sperm

concentration and ejaculate volume but if it becomes routine for bulls the semen production gets reduced. Then little longer time can be given for preparation e.g. Two false mounts and tying up for 5-10 minutes preparing another bulls during restrain, which will sufficiently stimulate the tied up bull and a higher amount of semen with better density can be expected. Change of teaser male also brings up desired effect.

Restraint :

False mounts and restraint of bulls increased the sperm out put. It has been observed by Hafs (1973) that if the bull is given as many as 3 false mounts the sperm out put increased from 7.1 to 13.5 billions and if the duration of active preparation is increased to ten minutes, the increase was to the extent of 16.8 billions. Kuefper (1973) is also of the same opinion.

Exercise and freguency of collection :

Effect of exercise : Beneficial effect of exercise on reaction time, ejaculate volume mass motility were observed by number of workers (Kozolo, 1967; Bhosrekar and Nagpaul; 1974). Paroshin and Obskalov (1976) reported increase in semen production by 35.7% by exercising bulls regularly. According to them exercise increased semen ejaculate volume, sperm concentration and motility.

Frequency of collection has not significantly affected the semen production either in Jersey or Holstein Friesian bulls (OH and IM, 1965; Singh et al 1968; Almquist and Amann 1968; Almquist et al, 1976 and Bhosrekar et al, 1980).

Frustration :

Frustration should be avoided. If the bulls are brought to the area of collection and are not collected, they get frustrated or disappointed and later on hesitate to mount. Therefore, it is always better to plan collection schedule in advance.

It can also occur due to faulty management e.g. young animals reared in isolation until maturity show abnormal sexual behavior These social isolation effects are permanent in males. Some individuals are much more seriously affected than others. In bulls sexual inhibition may get developed as a result of fear or repeated frustration at the time of semen collection. Some bulls become apprehensive for sudden changes of 'Pen', herdsmen or place of collection or semen collector. Some bulls refuse to mount when the collector stands too close to the teaser male. If the collector or attendants take part in operations e.g. blood sampling or disease testing etc. bulls refuse to donate the semen. Such inhibition can be removed by long abstinence of such bulls.

CHAPTER IV

SELECTION AND MANAGEMENT OF BULLS

Pedigree selection

It is said that "Judge the bull by its daughters and not by the pedigree", since even if the pedigree is good it is not necessary that it will have same transmitting ability to its daughters.

Since bull is half the herd,it is extremely essential to have careful selection of bull for breeding. It is possible to achieve success in dairy business by starting out with only average foundation cows, no advance can be made if the herd sire is selected in a similar fashion. Buying a scrub bull will prove most costly and unsatisfactory way of attempting to make progress in dairying. The most important Pedigree is the sire. If the sire is found to transmit high production, no consideration need to be given to his background. If the dam is a good producer herself there is no guarantee that she can transmit this trait due to limited number of calves she will produce in her life time.

Analysis of the performance of the sires' ancestors be carefully done. The feeding conditions, frequency of milking, length of lactation, butterfat percentage, all records should be converted to butterfat yield. Any physical congenital defects in ancestors should also be noted. Close relatives of the sire if available in the herd should be looked in for production records e.g. if daughters or sisters are available, their performance should be recorded. If sire index is available based on

daughters performance, it is the best otherwise dams performance or its sires dam's performance, or sisters performance should be taken into consideration for selection of the bull.

After doing selection from records, the bull's clinical history should also be noted (especially for fractures, traumatic reticulitis, hoof troubles etc.) vaccination etc., should be confirmed. The bulls should be tested for absence of (i) Brucellosis, (ii) Vibriosis, (iii) Trichomoniasis, (iv) Leptospirosis, (v) Listeriosis, (vi) Tuberculosis, (vii) Johne's disease. If the bull is free from all clinical or sub-clinical symptoms of any disease he should be examined for physical soundness.

Physical soundness

Testis : The feel of the testis should be pliable and soft. Hard testis indicate degenerative changes and/ or fibrosis of paranchyma. The size should be 12 to 15 cm. long 4 to 5 cm broad and 2 to 3 cm thick. It is very essential to know the normal size of the testis so that testis with hypoplasia can be avoided.

Epididymis : One should be able to feel the caput, corpus and cauda epididymis distinctly from testis. Fusion or hardness in the epididymis indicates organisation of cells because of degenerative changes due to fever etc. While examining these organs possibilities of segmental aplasia, spermiostasis should be kept in mind. Bull suffering from such defects should be discarded.

Spondylosis : The bulls should be allowed to mount before final selection so that physical defects like spondylosis traumatic reticulitis could be detected.

Normal curvature of the body at the time of mount and thrust will be absent in cases of traumatic reticulitis because of pain in chest and bulls would not like to mount in cases of spondylosis because of pain in back.

Locomotar system : The locomotar system, especially. The skeleton, joints and muscles of rear limbs and back, requires special attention. Mounting and balancing all weight on the rear limbs is associated with a considerable and sudden strain on the locomotar system of the region. Any defect might lead to temperary or permanent impairent of mounting ability of the animal.

Spastic Syndrome : The disease is seen in both cow and bull irrespective of breeds. It rarely happens below 4 years of age. The causes are not known but it certainly is associated with disturbed functions of central Nervous system, probably its spinal cord. Muscular spasms occur. Observation of bulls for longer period may be needed for detection of spontaneous attack.

Articular involvement : Straight hocks are undesirable conformation of joints. Any painful condition in joints may cause difficulty in mounting.

Hoof : The most common site for pathological lesions, which cause lameness in cattle, is the hoof. Hooves should be carefully examined for presence of begining footrot, penetrating wounds, corns and abscessess.

Badly outgrown hooves often result in permanent deformity in fetlock and digital joints. Trimming of hooves help in bulls restoring their libido.

Other defects : Other defects like torsion of testis, upward diversion of penis, cryptorchidism etc., should be avoided.

Internal organs : Rectal examination should be carried out for feeling of accessory sex glands. After these tests the bull should be allowed to give one natural service in a cow and three collections in artificial vagina in order to study its reproductive behaviour, libido etc.

Sperm defects : The bull should have proper penile movements, search for vagina and proper thrust. The semen of the bull should be examined for volume, mass activity, initial motility on dilution, live/dead count, head abnormalities and acrosomal defects. Colour of the semen should be normal cream or milky white colour, abnormal colour like brown or dark should be immediately discarded, it indicates haemospermia or deep bleeding in reproductive tract. Less than 15 per cent abnormal spermatozoa are within physiological limits, but too many abnormal spermatozoa of one type indicate something alarming. (e.g. pyriform heads or dag defect or coiled tails). Loose heads indicate degenerative changes in testis. Head abnormalities are of primary origin, so more attention should be given to count head abnormalities. After looking to all these above noted points, the selection of bull should be done.

Management

Housing : Most of the bull centres provide loose boxes to the bulls. Each box of the size of 10' x 15' with tiled or cement concreted roof. The floor for standing is cemented with antislippery device drains for urine and dung are usually provided. The enclosure walls

are half raised to allow free air movement and avoid dampness in the box. In European countries the sheds are centrally heated except in summer. Sufficient ventilation, water and comfortable floor and roof are the criteria of good animal house. The building material depends upon the availability of funds. Tiles or asbestos corrugated sheets provide good roofing material. A false sealing of plywood painted from inside with black paint will give additional comfort to the animal. The front wall should be provided with window of 4' x 2' with wire mesh fitting to avoid entry of mosquitoes or flies. Similarly on the back side similar sized window and an entry door is required with wire mesh fittings. Floor should be antislippery connected with urine and dung channel. The channel should not be too deep. Where the bulls are not tied the channels are not required but outlets should be provided in one corner so that after washing the floor, the wash water can be drained out. The manger about 2' high and 2' broad and 1' deep should be provided for the bull. To avoid the bull's standing in the manger, pipe at the height of 1.5' above the manger should be fixed so that bulls can eat through the space between pipe and the manger, at the same time they will not be able to stand in the manger and the wastage of the fodder will be minimised. A water trough should also be provided in the box.

For exotic bulls a shower should be provided with a fan so that the bull will feel comfort. In case he feels heat he will stand under shower at his will. Fan will remove the humidity and provide air to cool his body surface. Loose housing is preferable over tying. In latter method sufficient exercise is required to be given to bulls. The bulls should be provided with soft bedding in winter to avoid the frost bite in northern

India, while other places it is not essential. Buffalo bulls need special care in all the seasons. Buffalo is a very delicate animal and is affected by both hot and cold climatic temperatures. In winter it requires covering and warm bedding and in summer it requires showers or wallowing to have optimum semen quality and quantity.

Exercise : While it would be generally accepted that exercise is important for general health, bulls at many insemination centres have remained in good health and maintained a satisfactory level of semen production and fertility when allowed only the freedom of their boxes. Forced exercise in mechanical exercises for periods increasing from 15 to 30 minutes daily did not influence significantly on the quality or quantity of semen as compared to controls. This may not be true when bulls are tied continuously instandings. Ankylosis of Vertebral joints (Spondylosis) was demonstrated in 31 out of 37 bulls. Reference is also made to "standing disease" in which spastic hindleg condition occured which may be associated with spondylosis. These conditions are reported by Milk Marketing Board, England and Wales (1957) and Roberts (1953). A beneficial effect of exercise has been reported by Bonadonna (1956). Sorensen and Hansen (1950) reported that exercise improved semen volume and density when bulls were exercised 45 minutes prior to collection. Bhosrekar (1973) reported beneficial effect of exercise for Zebu bulls.

Handling of bulls : Since a great variation is observed in temperament and behaviour of bulls, a rigid system can not be laid down and be expected to be equally satisfactory for all bulls. The bulls should be washed,

groomed before collection to remove the loose hair, dirt from the body of the bull. All these practices will set conditioned reflexes in bulls. The set practice should be strictly followed.

Feeding and rearing of bulls : Current evidence indicates that dairy bulls should be well grown early, avoiding in so far as possible, damage to the testicular tissue from any cause. Sperm cell production is dependent on the amount of actively functioning tissue of the testis.Testis size, in turn, is directly dependent upon the size of the bull.

Adequate feeding of energy, protein, minerals and vitamins is necessary to promote optimum growth and development of young bull. The reproductive functions of young males may be depressed more by nutritive deficiencies than that of older males.

The onset of puberty is delayed markedly by a deficiency of energy or protein in the ration. Vitamin A is equally important in maintaining the integrity of germinal epithelium. In the several studies on the effect of feeding levels on reproductive performance of bull calves, it was evident that slowing the rate of growth delayed the onset of puberty. The effect of age and body size on the ability to produce semen has long been recognised but has not been considered extensively until recently. Number of factors influence the age of puberty but one amongst several is feeding.

Health control : Regular testing for brucellosis, campilobacteriosis, trichomoniasis, leptospirosis, listeriosis, tuberculosis and Johne's diseases should be carried out. Doubtful or positive reactors should be immediately culled. Treatment of minor ailments

should be carried out and recorded. Febrile diseases will hamper the quality of semen, so they should be attended immediately. e.g. thileriosis, foot and mouth etc. Because of the high temperature, temporary degeneration of seminal epithelium takes place and spermatogenesis goes defective. Foot and mouth causes sterility in bulls especially cross-bred as well as exotics for 2 to 3 months. Even on recovery from foot and mouth the resumption of semen donation takes longer time. More of loose heads are seen. There is loss of libido too. Sufficient rest should be given after foot and mouth attack before the bulls are collected (Bhosrekar, unpublished) ; Sharma (1967).

Any sort of stress on the body may cause deterioration of semen quality. e.g. transport stress (Pellerzi, 1975, Jaskowski and Szule, 1969). Febrile conditions in diseases like Foot and Mouth (Sharma and Sane, 1972), vaccinations against Rinderpest (Narsimhan et al. 1970), Foot and Mouth (Saxena et al. 1976; Tripathi and Saxena; 1976; Venketeshwar and Jagannadh Rao, 1970, Narasimha Rao, 1974). The percentage of abnormal spermatozoa goes up. The ejaculate volume and motility is markedly affected. Mid piece abnormalities like cork screw mid pieces appear in semen following ephemaral fever (Burgess and Chenoweth 1975). Heat stress also affect spermatogenesis. Skinner and Haow (1966) observed that exposure of Friesian and Afrikaner bulls above 40°C temperature stress for 12 hrs. damages the seminiferous tubules. Spermatids exhibit vaculation, and there is decrease in motility and live sperm. Similarly classical experiment by Legerlof of insulation of scrotum caused higher testicular temperature leading to disturbances in spermatogenesis. Loose heads, abnormal sperms appeared in semen while

removing the stress factor the semen came to normal after 6 weeks to 2 months.

Any febrile condition in bull causes damage to germinal epithelium and hamper the sperm production. Conditions like diarrhoea, bloat, foot rot, lameness and similar other problems cause bulls discomfort resulting in loss of semen production. Bulls suffering from lung infection, pain in chest also hesitate to mount. Even if such bulls mount they hesitate to give ejaculatory thrust causing losses.

CHAPTER V

REPRODUCTIVE BEHAVIOUR OF BULLS

Reproductive behaviour of bulls is influenced by both external as well as internal factors.

External factors : Include the animal's response to its social environment e.g. presence and behaviour of other animals particularly of its own species can exercise great influence on the behaviour of the individual. Some communications depend upon chemical senses e.g. those of taste and smell. Pheromones are the chemical substances excreted by one animal influencing the behaviour of another. Auditory communications are no less important in the mediation of reproductive behaviour e.g. bellowing or a call for mating attracts the male towards female. An obvious mode of communication is the visual one. The changing photoperiod, the ratio of light to dark per day is yet another external factor affecting behaviour. There is little doubt that principally it is this phenomenon which promotes seasonal breeding. However, rhythmic seasonal activity is often considered to depend upon internal factors as well as external stimuli.Harker (1964) has shown that such rhythm is controlled by an internal biological clock as well as input from environment.

Internal factors : The nervous system with its peripheral and central parts is the principal internal force in behaviour. It is the nervous system which affords great complexities in behaviour phenomenon. Although pituitory is the principal endocrine gland in the body, much of the control of this gland is invested in the hypothalmic region of the brain. Most of the

endocrine glands through out the body are involved in the production of hormones which are associated with behaviour of reproduction, but the pituitory gonadal axis is singularly important.

Tactile : Some genital stimulation takes place in female prior either to oestrus or to mating as a result of exploratory investigation by the male. The nosing of vulva by the male is seen so typically at precoitus. Vigorous nosing of vulva at the region of its ventral commissure is seen in the goat, cattle and sheep as well as buffalo.

Auditory : In moose the use of voice in oestrous female is widely noted. It is surprising that an animal, so singularly solitory in its habits should be dependent on the telereception of auditory signals between the sexes for mating to occur. It is clear that male and female moose in rutting season summon each other over great distances by their calls. Bellowing in cows and buffaloes has been noticed during oestrus this may be considered as mating call for a male. This typical bellow can be distinguished by the farmers from usual bellow at the milking hours for concentrate or calf, etc. Especially buffaloes bellow violently and become restless when they are in heat.

Visual : Visual stimuli perhaps play a greater role in directing reproductive behaviour than any other single form of stimulation. This is evident in deer for example antlers are carried by one sex only during breeding season.

Olfactory : With mounting evidence it becomes clear that olfaction is fundamental in the stimulation of

reproductive responses. Substances eliminated in urine are specific for reflex, since it is almost invariably induced after the subject has odour tested freshly voided urine particularly from the female. It is known that urine carries break down products of hormones including reproductive hormones and it is reasonable assumption that oestrus cycle phasing may be recognizable to male animal by the odour testing of urine. Odorous substances, eliminated by the animal have a specific stimulatory effect on the animal of opposite sex, are termed pheromones. The role of pheromones in reproductive behaviour is considerable e.g. Preputial fluid is medium of pheromones in boars (Dutt et al, 1959; Signoret and Dumensil Du Buissou 1962 a, b.). Scent is not only chemically different for all genotypes but also changes in the individual. A particular musk like smell characterises the reindeer stag during rut (Meschaks and Nordkvist, 1962).

Photoperiod : It has been known for some time that light is a cause of reproductive periodicity in animals (Rowan 1926). Seasonally breeding ungulates, respond to photoperiodic stimulation in most cases. Where season influences reproduction, the photoperiod is usually the principal seasonal factor.

Some animals exhibit their maximal reproductive responses during the period of the year which provides long day light. Horses are in this category. A variety of other animals show their full mating behaviour in the proportion of the year which has short day light. Ovine and Caprine animals are in this category (Sykes and Cole 1944; Hart 1950). It is understood now that stimulation of gonads by photoperiodism occur following pitutory stimulation via the hypothalamus. Hyphophysectomised animals do not respond to light

breeding stimuli but go in state of anaestrus (Marshall 1942).

Environment : Transportation adversely affects the mating behaviour of male animal. It is recognised that transportation can immediately reduce the sex drive of a subject to a point of impotence. Competition between males appears to exaggerate the reproduction responses of some males. Most notably those reaching a peak of maturation, where as it has an inhibiting or subduing effect on aging males. Rutting species of many types show that as social pressures increase older male animals are more likely to make a total withdrawal from community (Altman 1963 b).

Cerebral cortex : The cortex possesses four main sensory areas into which sensory projection fibres discharge (1) the somanaesthetic or body sense area, (2) the visual area, (3) the auditory area, (4) the olfactory area. All of these are important in receipt and interpretation of nerve signals and are fundamental in determining reproductive behaviour.

Experimental factors :

(a) Learning : The simplest form of learning is the adoption of responses which are appropriate and suited to their stimuli. Higher animals such as ungulates apparently learn a great deal by observing the behaviour of other animals, with which they are in association. After observing the behaviour in others, they may attempt to respect the actions themselves. This is learning by imitation.
(b) Conditioning : Instinctive machanisms or inherited responses to stimuli : Bane (1954) has shown that in

mature bulls, the sexual behaviour is of genetic origin. Goy and Jakway (1962) are also of the same opinion. It is also important to recognise that even with this fixed foundation, instinctive behaviour can be modified by experimental factors and by learning and that environment can operate against heridity (Lehren 1953, 1962).

(c) Release of innate responses : Tin Bergen has described the existence of "Innate releasing mechanism" (IRM) for conditioning the animals for certain reflexes.

Seasonal and climatic breeding responses : "The breeding season of most of the species appears to be controlled not by a single factor such as periodicity but by a combination of external stimuli including behavioural ones, which vary in different species but nevertheless act through sense organs upon internal rhythms of the individual. In vertabrates this mediation operates through exteroceptors. The CNS, the hypothalamus and anterior pitutory, which secretes the appropriate gonadotrophins" (Cloudsley Thomson 1961). Diurnal variation in mating behaviour is noticed amongst several species some prefer day time while others are specific noctoranal breeders. Noctornal breeding is a rule in swamp buffalo (Bubalis bubalis) Asdell, 1946). McGregor (1941 and 1950) also reported this fact and observed that by contrast, the river race of Asiatic buffaloes practised day time mating. The Sambar (cervus unicolor), the largest Indian deer, has noctornal habits and during the rutting season the stags are active at night and their loud calling resounds particularly during darker hours. Basirov (1960) observed that bulls and buffalo bulls exposed to high temperatures during the summer period in Azerbaijan showed marked reduction in

libido particularly when heat stress occurred. He noted that the libido was inhibited in all buffaloes and greatly reduced in bull in air temperature of 40°C to 50° C. These effects were transiant and libido could be restored after wetting the bulls with water. Bhosrekar (1974) observed that buffalo bulls could be collected even in summer with good quality semen when collected early in the morning. Slight rise in temperature above 30° C could cause futile collection. Once in a week per bull through out the year if collected between 5.00 a.m. to 7.00 a.m. yields satisfactory results as far as semen ejaculate quality is concerned. The same author recorded that there is more percentage of abnormal ejaculates during winter from buffalo bulls. This is also confirmed by other workers (Sengupta et al 1963; Abhi et al 1968). Hafez (1953) observed that water sprinkling acts as a stimulant to those buffalo bulls whose libido is reduced during summer. The same type of behaviour was recorded for exotic bulls stationed in India. It is author's personal experience that bulls of Brown Swiss, and Holstein breeds lost their libido completely because of summer stress at Karnal while the sons of these exotic bulls born and brought up at Karnal gave semen collections in summer too, though of poor quality. Jersey bulls did not show such effect. At Uruli-Kanchan (Pune) the summer stress was minimum. The bulls of Holstein-Friesian and Jersey breeds did not show any deviation from normal behaviour. A summary of the effect of ambient temperature on libido has been provided recently by Yeates (1965) who states that high ambient temperature per se is without effect on libido at the endocrine level but that it has secondary effect on libido by diverting the male from sexual interest when a state of discomfort or anxiety, induced by high temperature is reached.

Service behaviour is one of the important measures for assessing reproductive soundness in bulls. Patterns of male sexual behaviour have been described by Hafez and Bouisoou (1975) which encompass sequential behavioural elements like sexual interest, erection, protrusion, mounting, body positioning, type of seeking ejaculatory thrust and dismounting. Manifestations of these patterns in bull varies from full to no expression.

Bhosrekar et al. (1988) found no significant difference between breeds for different components of service behaviour, however they showed seasonal variation significantly for reaction time Sharma et al. (1982) and Bhagoji (1988) also recorded seasonal variation in reaction time in HF and Jersey cross-bred bulls. Bull to bull variation for behavioural components was so significantly high in each breed that effects of breed and season were not seen (Bhosrekar et al 1988), Ali et al (1981) also reported highly significant variation between bulls for mounting behaviour.

Normal copulation consists of a sequence of behavioural elements viz courtship, erection and protrusion, mounting, intromission, ejaculatory thrust and ejaculation and dismounting. These elements are regarded as typical male behaviour. The manifestation of these patterns in the bulls vary from full to no expression.

Intromission is the act of inserting the penis into the vagina, copulation (coitus) is intromission culminating in ejaculation of semen. Ejaculatory thrust is the act of forcing the inserted penis further into vagina just before ejaculation.

Courtship : It is more evident on range conditions rather than restricted conditions. The bull detects the proestrus cow and remains in general vicinity till the oestrus appears. This behaviour is termed as "guarding" by Schloeth (1961). During guarding the bull may mount several times showing partial erection and protrusion and perhaps dribbling of accessory fluid. These mounts are usually unsuccessful because the female does not "stand".

As the oestrus approaches the bull follows her closely and gets more excited. He licks and smells her external genetalia curls upper lip showing gum region, while the head holding up, nares get distended. This posture is termed as Flehman, by Schneider (1931). The bull often shows pawing and horning the ground; throwing soil on his neck and withers, and rubbing his head on ground. This behaviour is generally shown by the bulls in order to threaten and chasing away young bulls (Kerriush).

Chin resting : It often appears preceeding to mounting. The bull follows the cow and stands behind the cow, raises his head, so as to contact cows rump with his chin and throat. In fact, the bull puts mild forward pressure with the chin and throat and pushes the female with the chest. This behaviour resembles to the neck thrusting behaviour seen in other ungulate species like Indian black buck, Uganda Kob, to drive oestrus females to specific mating territories or arenas. The non receptive females escape chin resting and avoid the male. The oestrus females respond the chin resting pressure by "standing" or even exerting back pressure; the bull then mounts.

Copulation : It is quick process as compared to horses or dogs or swine. In mounting, the bull quickly

shifts its weight to the hindlegs, lifts his shoulder and forelegs off the ground and move forward to straddle the cow near the middle of her back. The penis is near the vaginal orifice. It is free and sways to and fro until glans reaches and penetrates the vagina. After further interomission the cow's vaginal sphincter contracts around the penis. As a result of sudden contraction of bull's abdominal muscles, while the forelegs are "fixed" on the cows pelvis, the bull's pelvic region is quickly brought into direct apposition to the cow's genetalia and maximum intromission leading to ejaculation is achieved. The bulls even lift the hind legs and jump and bring the pelvic region in apposition to genetalia of cow and ejaculate.This gives an appearance of active leap. The ejaculatory thrust is given with maximum vigour and semen is ejaculated as a single violent gush near the os cervix. The abdominal muscles then relax and the bull dismounts slowly. In this process the bull generally draws his jaw across the back of the cow. This action has been taken as a basis of detecting cows which have been served. A crayon block attached to the lower part of strong leather head collar of a bull marks the cow as he dismounts. This can be advantageous for detecting heat (Yeates; 1965).

In camel, the mating behaviour has been described by Singh and Prakash (1964). The male Camel grinds the teeth. It extrudes its soft palate through the mouth, circles the female, he pushes his neck on the neck of female camel, bites the shoulder of the female. The behaviour leads the female to assure a seated posture. The male camel assures similar dog setting position behind the female. The male extends the foreleg to grip the female and effects intromission. The entire copulation takes 15 minutes.

In sheep and goats the male noses the vulva, paws with forelegs, bleets, stamps with forefoot, licks rapidly. A very quick intromission with strong clasp by forelegs and single thrust. The male mounts several times the oestrus female, while some of the mature rams serve only once.

It has been assumed that sense of smell plays an important if not primary role in sexual stimulation of a bull. Hart et al (1946) indicated that olfactory cues are all important in arousal of sexual behaviour in cattle on the contrary Hale (1966) proved that visual stimuli are more potent than olfactory. Males provide the same degree of stimulation as females (Almquist and Hale, 1956) and restrained anoestrus female as stimulating as oestrus animals (Prabhu 1956). It is common practice to use anoestrus female or castrated or uncastrated males as teasers for semen collection.

Masturbation

It is commonly seen in bulls used for semen collection. The bull arches his back takes in and out his penis out of Prepuce, which serves tactile stimulation and eventually causes ejaculation (Hultnas, 1961).

In some cases the contraction of abdominal muscles is so much so that hind legs cross the forelegs and bulls loose their balance. Hultnas (1961) has also shown more frequent masturbation on high protein diet. The mucosa of penis becomes more sensitive to tactile stimulation as a result of these diets. Bhosrekar and Razdan (1973) also recorded similar finding causing deterioration in semen quality.

Frequency of ejaculation

Frequency of ejaculation under natural conditions varies with climate, breed, individual, nutrition, period of sexual rest and nature of sexual stimuli (Hafez, 1968). In favourable climatic conditions, time of day or night has no bearing on sexual activity. Sexual activity is restricted in excessive heat or cold conditions. The usual practice at A.I. Centres is to restrict each bull to one or two collections per week.

Libido

Libdo or sex drive can be measured by several indices designed to guage the intensity of bull's sexual behaviour.

The most meaningful measurements of sex drive are

1. The number of ejaculations during a constant period of time or until no more ejaculations can be collected in a reasonable period of time with all available stimuli.

2. Reaction time, that is the time taken by the bull to mount and ejaculate when brought near the cow or stimulus animal in unstimulated conditions.

3. Measurement of the recovery time after stimulus satiation provides critically important information relating to the level of behaviour.

Lanc et al. (1983) observed significant effect of rearing environment on serving behaviour of young beef bulls. They found that mean serving efficiency as well

as mean serving capacity was greater for individually penned bulls than for group penned bulls.

It has been our observation that newly introduced young bulls as well as old bulls take longer time for dismount while bulls who have been in collection for quite some time quickly dismount after ejaculatory thrust. In buffalo bulls the thrust is no doubt vigorous but the buffalo bulls are not seen lifting hind legs and jumping for giving thrust as in case of cattle bulls. They slowly dismount unlike cattle bulls and take more than a minute in some cases.

Factors Affecting Libido

Genetic factors : Olson and Peterson (1951) and Bane, (1954) have vividly proved with Monozygotic twins that libido is genetically governed and is fairly constant for each bull. The latency of ejaculation or the number of ejaculations in a given unit of time are highly repeatable.

Bulls of exotic breeds rapidly mount anestrus cows or other bulls, where as bulls of Zebu breeds exhibit a well marked sexual sluggishness and mount only the cows with full oestrus (Anderson, 1948). Donaldson (1962) found less number of calves coming from Brahman cross bulls as compared to European type bulls when equal number of them were turned out in Hereford herd showing longer reaction time or less libido in Brahman cross-bulls. Bonadonna (1953) reported variations in sex drive amongst European breeds. Shorthorn and Guernsey bulls react more slowly than Friesians, while amongst Friesians differences were noticed in those coming from USA, Holland or Germany.

Plane of nutrition : Low plane of nutrition for a longer period of time reduces the libido in bulls. This may be due to physiological stress causing debility.

Climate : High climatic temperature reduces sexual drive. This is especially true for buffaloes and exotic bulls in tropics. Summer temperature causes less sexual drive in above mentioned types. Early collections before sun rise are generally fruitful.

Exercise : Exercise to the bulls keep them trimmed and in good physical health. It reduces reaction time if given just before collection (Bhosrekar, 1973). Overfeeding causes drop in semen quality and bulls if not given exercise get fatier, sluggish.

Age : It is generally noticed that younger bulls are more active and sexually alert but as the age advances the libido goes down. The prime period in Bulls life is 15 months to 62 months, in exotic breeds, and 36 months to 84 months in tropical breeds.

Diseases : Diseases of systemic origin, fever or debilitating diseases cause varying degree of loss of libido. Injuries or diseases of penis, prepuce or testis can also reduce libido because of physical inability of bulls to mount.

Traumatic reticulitis or spondylosis or nail in the hoof or over grown hooves also causes loss of libido because of pain experienced at mounting.

Stimulus pressure : Hale and Almquist (1960) reported that the recovery from sexual satiation to a particular stimulus varies from individual to individual those which quickly recover show less reaction time.

Conditional stimuli like sound of gate opening, whistling, often contribute to enhance the stimulus pressure. Precoital restraint, false mounts, changing the teaser etc., cause higher excitation and works as stimulation for ejaculation.

CHAPTER V I

COLLECTION OF SEMEN

Techniques of semen collection :

One of the most important aspects of artificial insemination is the proper and clean collection of semen. This involves the proper scheduling of bulls and sexual preparation as well as techniques of semen collection.

Frequency of semen collection should be determined depending upon the age and body size of the bulls. Very young bulls to start with should be collected only once in fortnight. Slightly older bulls (between 2 to 3.5 years) of age should be collected once in a week or even twice a week if they maintain the quality of semen and the bulls above 3.5 years until 7 years should be collected only once in a week. This schedule is applicable to exotic or cross-bred bulls but tropical breeds have late maturity and therefore, the bulls and buffalo bulls donate semen around 36 months (Bhosrekar 1975) some of the bulls of dairy breeds like Tharparkar, Red Sindhi have donated earlier than 3 years.

Preparation of bulls for collection consists of washing and grooming of bulls, which may form the source of conditioned reflexes for collection. Tying of aprons to the bulls (the apron is 65 x 62 cm. with semicircular cut at one end by leaving 12 cm. on either side and fixing slightly heavy 2 to 3 metal pieces on opposite side to keep it straight and to avoid folding of apron). The apron cloth should be of coated rexin. The rexin side

should face the penis of bulls. The apron should have two metallic rings on each corner fixed up and rubber strap going round the heart girth of a bull. The apron should be fixed behind the elbows and should hang so much so that it does not touch ground while standing. (Photo 1)

The tuft of hair on the preputial opening should not be clipped and should be allowed to grow since this is the natural barrier which will prevent entry of foreign particles in prepuce. The clipping of hair will cause irritation and stimulation for glans penis resulting in habitual masturbation.

Photo No. 1

The bull is allowed to mount the teaser with the apron on so that it prevents penis coming in contact with the hind portion of the teaser thereby preventing contamination. The bull should work on the teaser and then slowly dismount. Two to three false mounts in 10 minutes improves the ejaculate volume and number of sperm per ejaculate (Hafs ;1973).

Advantages of using bull apron :

* It prevents cross infection from bull to bull, which otherwise may take place through contact of penis to the rump of the teaser.

* It prevents losses of semen ejaculates which happen in certain bulls by tactile stimulation because of the penis rubbing against the skin of the rump of teaser and bulls throwing semen out side before penis is directed in A.V.

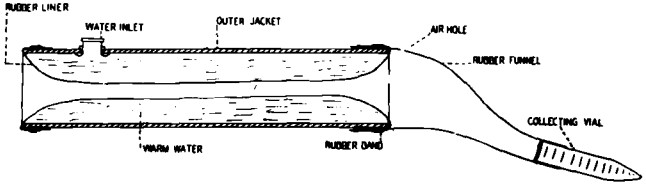
* The Apron prevents the direct contact with the skin of teaser and thereby more hygienic ejaculates can be collected.

Photo No. 2

Artificial vagina technique :

The best method of semen collection is with artificial vagina. Walton (1945) preferred short artificial vagina. There are three universal sizes of artificial vagina 25, 30, and 40 cm. The artificial vagina is a rubber cylinder with a water inlet and air inlet valves. The

rubber cylinder has rims on either side a combined water and air inlet is provided about 8 cms. from one end. The rubber lining either of latex rubber or PVC is mounted on the cylinder and is fixed with the help of rubber strap. Latex rubber cone is mounted on the cylinder to the end where water and air inlets are closer. The glass semen collection tube is fixed to the conical end of rubber cone and a insulation bag is mounted on glass tube and rubber cone and fixed on the rubber cylinder with strap. The water of 55° C is filled in through the inlet valve. The AV is then shaken and the water is discarded (pre-warming). The water of correct temperature 48° C is filled in. The A.V. is now ready for semen collection. No lubricant should be applied to AV. Fig. No. 17



DIAGRAMATIC ARTIFICIAL VAGINA FOR THE COLLECTION OF BULL SEMEN

The air should be pumped to give a star like appearance. At BAIF two types of AVs are used one smaller 25 cm and longer 30 cms. Smaller AVS are used for young bulls and buffalo bulls while longer

AVs are used for adult cattle bulls. Normally the AV's final temperature is kept at 48° C with little variation in summer and winter. In winter the temperature is raised to 49°C while in summer it is reduced to 47° C. Some bulls always desire to have higher temperature. Two types of rubber linings are used. With old bulls not donating semen in smooth rubber lining rough linings are used. Troublesome bulls refusing to donate semen do donate semen easily in Artificial vagina with rough lining. In young bulls usually smooth rubber lining AVs should be used. Polyethylene linings and cones for AV proved to be the most useful from the point of view of quality of semen (Flick and Merilan; 1988).

Since the semen comes out of reproductive tract of male at certain specific temperature (i.e. 35° C) the collection tube should be kept at 35° C so that freshly ejaculated spermatozoa should not be put to thermal shock. This can be effected by providing a small plastic jacket for collection tube containing warm water at 35° C or felt cap. As soon as the semen is collected it should be then placed in a water bath of 35° C. The collection tubes are kept warm at 35° C by use of warming table and the felt cap is mounted on the tube to retain the warmth of tube. The insulation bag is also mounted on the cone and tube. On an average 50 mm Hg pressure is given in AV, using cycle pump for pumping air in AV. Some bulls like to have more pressure. The pressure ranges from 45 to 55 mm. Hg (Bhosrekar et al, 1988).

The method of collection

The semen collector should have a blue greenish or green overall on himself. He should wear gum boot

with uncrushable toes. He should also wear hand gloves.

He should hold the artificial vagina with water and air inlets pointing to the ground in a inclined position of 45°. When the bull mounts the teaser, he should push the bull with his shoulder catch hold of sheath and allow the protruded penis to search the A.V. As soon as the penis touches the warm surface of A.V. he will give a strong thrust by virtually lifting his hind legs and bringing his hind portion in apposition to the teaser's hind portion and ejaculates. The A.V. should not be removed immediately but the bull should be allowed to dismount and then the A.V. should be removed. Immediately the A.V. should be held horizontally and not vertically to avoid the contamination of semen by preputial exudates and flora. The AV should not be carried inside the lab, but only semen should be delivered in and A.V. should go for washing. Some scientists like small A.Vs to be used (so that ejaculates can be directly had in rubber cone).

It avoids :

* Warm surface of A.V. and thus thermal shock
* Preputial contamination from A.V. - generally small young bulls should be collected in small A.Vs while large bulls should be collected in bigger size A.V.

The bulls prefer thick lining and pressure. The thin smooth linings do not last long on giving pressure and on sterilizations and they give way on thrust by the bull. Thus the bull gets his penis burnt by warm water in addition to this the semen gets spoiled and the bull gets shy for mounting second time.

A.V. method of semen collection holds good for bucks, Rams, Boars and Stallions. The size of A.V. differs from species to species with little modification in construction of A.V. e.g. Bucks require A.V. of small size 20 cm., the diameter also is small, linings and cones also are short to the size of A.V. The A.V. for stallions is 54 cm. long and diameter is 13 cm. The diameter is reduced at distal end and is only 8.5 cm. To distal end rubber cone and semen collection bottle is fixed. In Denmark and other Scandinavian countries condom has received a support. (Rasbech 1959). For Boars, short A.V. which is used for goat can be used with intermittent air pumping to supply full pressure simulating the locking device. Only cone holding the protruded penis with fingers will also allow the boars to ejaculate since the finger can act as screw type locking device.

Electro - Ejaculation

This method is preferred in males that refuse to donate semen in artificial vagina or when injuries or infirmities make this impossible. This method of semen collection is justified in cases where the cause of inability to mount is non-genetic.

The electro-ejaculator is run on 110 volts as well as 12 volts dry cell. The new models are transistorised and portable. For bulls the rectal probe is either ring or straight electrodes. The bull has To be backrecked before this is applied since faeces in the rectum prevents proper stimulation. The erection is usually present with electrode method and semen can be collected with contamination from prepuce. Secretion from accessory sex glands takes place at lower voltage level and ejaculation at higher voltages.

CHAPTER VII

EVALUATION OF SEMEN

Methods of evaluation include gross evaluation, Microscopic and Chemical.

* Gross evaluation

Include the visual appraisal of semen. The colour, consistency, volume etc.

* Microscopic evaluation

Includes examination of semen for mass activity, initial motility, live/dead count, total count, abnormal count etc.

* Chemical evaluation

Includes estimation of pH, methylene blue reduction time, initial fructose content and fructolytic index, oxygen uptake, Resazurin test, G O T, hyaluronidase and catalase test. Blom (1955) considers catalase test as a measure for detecting bacterial contamination, of semen sample.

Gross evaluation :

Semen evaluation should be as rapid as possible so that the semen sample collected is processed further for preservation in shortest possible time, so that it maintains its initial quality.

As soon as the semen is delivered in the laboratory the volume of semen should be noted to the nearest of 0.1 ml from the graduations provided on the tube. The colour and consistency is also noted simultaneously. Abnormal colour are other than cream or milky white and lemon for bulls and chalky white or milky white for buffaloes. Bucks also donate milky or thick creamy or lemon coloured semen.

The normal volume of semen for different classes of livestock is as under :

Bulls	5.0 to 8.0 ml.
Bucks	0.2 to 1.2 ml.
Rams	0.8 to 1.2 ml.
Buffaloes	1.0 to 4.0 ml.
Stallion	60.0 to 100.0 ml.
Boar	150.0 to 200.0 ml. (gell free volume).

Individuals of each species may give as high as 20 ml. in bulls, 12 ml. in buffaloes, 4 ml. in rams and goats and 150 ml. in stallions and 500 ml. in boars but such males are exception. The lemon colour in the semen of some individuals is due to flavin compounds especially riboflavin, pigment. This colour is harmless and should be considered as normal. Abnormal color like reddish, brownish or blackish colour indicate bleeding of capillaries on the surface of glans or in seminal vesicles or deep parts of reproductive tract respectively, giving the semen respective colours. Haemospermia is generally the cause of brownish semen. Haemospermia may be due to tumour or cold abscess on seminal vesicles or any other part of reproductive tract pressing it and thus causing breakage of the capillaries giving brownish colour to the semen. The author has seen a case of

haemospermia in Holstein bull because of pressure of cold abscess on seminal vesicles. Density or consistency of semen should normally be thick opaque but variations are also noted ranging from the watery to thick creamy and opaque.This again depends upon the frequency of collection. The volume, and consistency changes according to frequency of collection body size and age, nutrition, exercise, teasing etc. (see elsewhere).

Microscopic evaluation :

Microscopic evaluation of semen includes normally two tests in routine i.e. mass activity and initial motility but other tests like live count, abnormal count etc., can be taken occasionally in order to know the functional integrity of testis. Mass activity can be graded in 0 to +++ grades or 0 to to +++++ grades. This is most subjective test and can vary from individual to individual. Most of the laboratories in world follow 0 to +++ grade. Where +++ means highest quality semen showing very strong eddies and waves ++ is one step below the highest i.e. the waves and eddies are present but they are mild and the force and vigour is not observed. The condition in between these two grades can be suitably graded as ++ (+). + or +(+) is poor quality semen. The eddies or waves are absent and movement of individual spermatozoa can be easily seen. Such a semen should be discarded. For dilution, semen with +++ and ++ (+) grades can be used.

Initial motility is graded in per cent spermatozoa having progressive motility. This is done on dilution with dilutor in the ratio of 1:10 in small test tubes before the semen is finally diluted. A thin drop of

diluted semen is kept on a warm slide and covered with a cover glass and seen under high power of microscope where phase contrast is not available. As a rule this has to be strictly observed under phase contrast microscope. The phase contrast microscope gives as fair idea about the percentage of progressively motile spermatozoa. 20 objective and 20 phase combination should be kept for examination. Before one examines in phase contrast the field has to be adjusted for giving clear one ring and not otherwise. This can be done by adjusting the phase with the help of gadgets provided alongwith the microscope (carefully read the instruction manual).

An approximate idea of dead and immotile spermatozoa can be had by looking the field under microscope and estimating progressively motile spermatozoa one can easily estimate approximately correct percentage of motile sperms e.g. if 10 sperms are taken in the field and out of which 8 are moving in forward direction and two are dead or immotile or having oscillating or circular motility. It can be taken as 80 per cent progressive motility. A good quality semen should contain 70 to 90 per cent progressively motile spermatozoa. 10 to 15 per cent of dead and/or abnormal spermatozoa having secondary defects are physiologically permissible. Any specific defect of spermatozoa should not exceed more than 3 to 4 per cent (M.M.B. Report 1975).

The types of motilities seen are oscillatory. This is normally seen in aged spermatozoa which have exhausted all energy and on verge of death. Circular motility is shown by spermatozoa which have suffered either thermal shock or osmotic shock due to dilutor not being isotonic. Kotaya (1975 personal

communication) recorded 50 per cent of Brown Swiss cross-bred bulls having low quality semen. Similar observations were recorded by Bhosrekar (1973) and Bhosrekar (1988) for other crossbreds. The live and dead differential count can be estimated by staining the semen with Nigrosin-Eosion (see elsewhere for composition and staining technique) and preparing the thin smear, which can be seen under oil immersion lens of microscope. The live sperm remain unstained while dead sperm take the stain. The results are highly correlated with the visual estimates of progressively motile sperm. Acrosome morphology can be studied by interference contrast microscopy or by Giemsa staining.

Sperm concentration :

Sperm concentration can be estimated by haemocytometric slide (Neubar'schamber) by diluting the semen in 1:200 dilution ratio. The procedure is the same as used for RBC count. The sperm cells are counted in 5 big squares of 16 small squares each or 80 small squares. For this 4 corner squares and one middle square is taken into account. A hand tally counter is useful for such counting. The total number of sperms counted in 5 squares or 80 small squares multiplied by 10,000 gives the total number of sperms present in 1 cu-mm. and again multiplying by 1,000 the total number of spermatozoa per ml. can be easily obtained. (Fig. 19)

The diluting fluid is 0.9 per cent saline solution with formaldehyde. The thoma Red cell pipettes can be used for diluting semen. The semen is sucked into red cell thoma pipette upto mark 0.5 and plunged into diluting fluid and the fluid is then drawn upto 1.01

mark just above the bulb. The pipette is thoroughly bumped against palm of hand and the contents of pipette are mixed properly. First few drops are discarded and the counting chamber is charged after placing the cover glass on the haemocytometer slide. After waiting for few minutes the counts can be taken under 300 x magnification and calculation done as above. (Herman and Madden, 1953) The normal concentration for bull semen is 1000 to 1200 millions per ml. (Fig. 19)

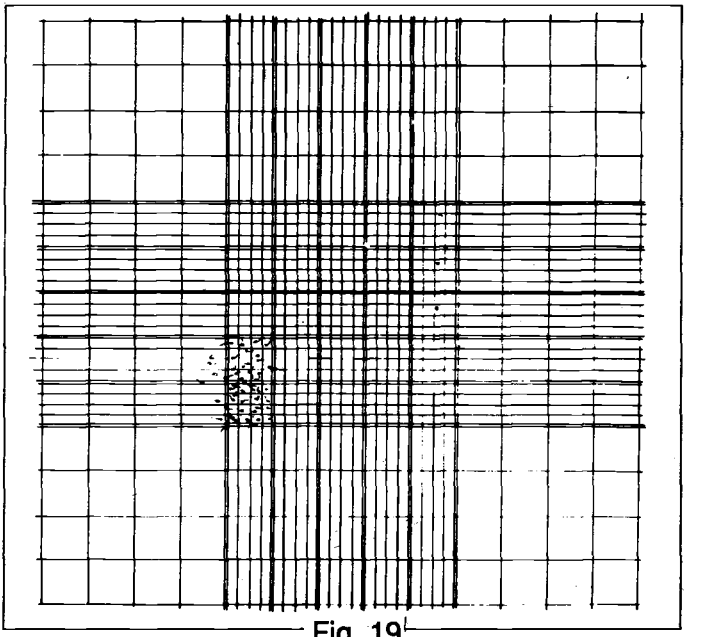


Fig. 19

Other and more practical method of estimating total count is by photoelectric colorimeter. Where quick determinations are required for routine dilution and preservation this technique is the best. The dilutions are done in 2.9 per cent sodium citrate buffer. 7.8 ml. of the buffer is taken into tube of colorimeter and to it 0.2 ml. of freshly ejaculated semen is added. This is

thoroughly mixed and the optical density or transmittance is recorded in EEL Photoelectric colorimeter with blue filter No. 303. The sperm concentration can be noted from the chart already prepared for each degree of transmittance. This standardization can be done from total counts on Neubar counting chamber (already described).

Chemical methods of evaluation :

* **pH** : pH of the semen can be tested approximately by taking a drop of semen in watch glass and adding a drop of bromothymol blue indicator and mixing it well by a bulb and capillary tube. The mixed content is drawn in capillary tube with the help of bulb and the colour is watched against a standard colour capillaries for different pH. The tube where exactly the colour matches is the approximate pH of semen. The capillary pH comparator is available with B.D.H. The other method is with the help of pH meter. The single electrode pH meter is most suitable for this purpose and most accurate estimations can be done with the help of pH meter.

The normal pH of bull semen is 6.2 to 6.8 while that of buffalo is 6.8 to 7.2 .Excellent quality semen gives pH of 6.4 to 6.5 while poor quality semen shows pH towards neutrality. Similarly in buffaloes good quality semen has pH of 6.8 while poor quality show 7.0 to 7.2 Since poor quality semen contains larger amount of fluid from urethral and accessary glands (Salisbury and Van Denmark, 1962). The pH of semen also decreases on time lapse between collection and measurement of pH since the fructose in the semen is broken down by spermatozoa under anaerobic condition converting it to lactic acid. The anaerobic

conditions are usually expected to exist in narrow collection tube. The pH is likely to decrease (Salisbury and Van Denmark, 1962).

* **Buffering capacity** : Buffering capacity is the chemical ability of a fluid to absorb acid or alkali with little change in pH. Bull semen is highly buffered at pH levels below 5.5 and above 9.0. It is only moderately well buffered at pH 5.5 - 6.5 and at pH 8.0 to 9.0. It lacks buffering capacity between pH 5.5 to 8.0 (Millett and Salisbury, 1942, Smith 1940) but depending upon initial pH, the addition of a constant volume of N/ 10 HCl to constant volume of semen results in markedly different pH decreases (Anderson 1946) because of the poor buffering capacity at pH 6.5 to 8.0. The buffering capacity of bull and human semen is similar. The semen of dog and stallion is virtually without buffering capacity (Millett and Salisbury 1942). Buffering capacity of buffalo semen is 1.40 pH units (Srivastava and Raza Nasir 1968). Margolin (1943) recorded 0.85 to 1.66 pH units for bull semen.

* **Methylene blue reduction test** : When methylene blue is reduced by the addition of two hydrogen ions it losses its deep blue colour. Reduced or leucomethylene blue is a white powder. Thunberg, a Swedish scientist has used this test in studies of cellular metabolism. Methylene blue, a hydrogen acceptor, receives the hydrogen ions from a hydrogen donor, a substrate, which has given up the ions by action of intracellular enzyme, a dehydrogenase. The reaction must take place in absence of air for the oxygen in air rapidly oxidises leucomethylene blue to methylene blue.

So under suitable anaerobic conditions the rate of hydrogen transfer in the metabolism of sperm cells may be determined from the rate at which methylene blue is reduced.

Nakano (1930) was the first Scientist to study the metabolic activity of sperm cells by using methylene blue. Later on number of Scientists have used this test for assessing bull semen quality (Sergin, 1940; Lardy and Phillips, 1941; Klein and Saroka, 1941; Sorensen 1942). Semakov (1963) and Hankiewiz et al (1964) observed strong correlation between dehydrogenase activity of semen, sperm motility, concentration of spermatozoa and live count. Upsenskii (1967) showed strong correlation between MBR time and fertility. Vasileva et al (1969) found succinic dehydrogenase activity to be a better index of fertilizing capacity of spermatozoa than motility index or percentage of live sperms. Oloufa et.al (1959), Salash (1972), Kodagali (1972) and Bhosrekar (1980) all have recorded lesser MBR time for 2 nd ejaculate as compared to I st presumably because of higher sperm concentration and massactivity. However, they did not notice any seasonal variation.

Procedure

* A solution of methylene blue is prepared by dissolving 50 mg. of methylene blue in 100 ml. of 2.9 per cent sodium citrate buffer.

* 0.2 ml. of semen is diluted in 0.8 ml. egg yolk. Citrate dilutor in a 10 ml. tube vial and thoroughly mixed (1:4 dilution rate).

* 0.1 ml. of methylene blue solution is added and mixed by tapping and bumping on palm of hand.

* The mixture is then scaled with 1/2 inch layer of mineral oil.

* Incubate in a water bath at 45^0 C.

* Observe the time required for losing the colour. A good quality semen should be able to reduce the colour in 3 to 6 minutes. The semen which retains the colour for 9 minutes or longer should not be used for insemination purpose because in such a samples the live sperms are very little.

Oxygen uptake : Walton and Edwards (1938) were the first to use the oxygen consumption rate of semen samples as a measure indicative of potential fertilizing capacity of spermatozoa. This can be done by Warburg's apparatus. Oxygen uptake by spermatozoa is measured by manometric determination. The sperm cells diluted in various dilutors are kept in small flask, at body temperature in a bath. The system is closed one, wherein the CO_2 produced is absorbed by an alkali. The O2 consumed and CO_2 produced can be determined. The composition of the diluent, pH, as well as the temperature of the diluent influences the O_2 consumption.

Fructolytic index : Fructolytic index is the amount of fructose used by 10^9 spermatozoa per hour at 37^0 C. This gives fairly good idea about the livability of spermatozoa and sperm metabolism. The sample of semen should be well buffered otherwise the

fructolysis will stop at certain stage and the results will be erroneous. Mann (1948 a and b) proposed a method which is as follows :

0.4 ml. sample of the semen to be used is pipetted in narrow glass tubes, to which 0.2 ml. of 0.25 M phosphate buffer is added (pH 7.4). A 0.1 ml. sub sample is withdrawn immediately deproteinised and its fructose content determined (The de-proteinisation is done with 2 per cent zinc sulphate 1 ml and 0.1 N NaOH 1 ml.).

The remainder of the sample is incubated at 37° C. for one hour. The sample is then withdrawn as per at 'O' hour. The fructose is determined. The difference between 'O' and 1st hour readings will be the fructose utilisation.

The fructose utilisation is done with sub sample of 0.1 ml. after deproteinization by adding 1.9 ml. water 1 ml 2 per cent zinc sulphate and 1 ml. 0.1 N NaOH. The mixture is heated for one minute in a boiling water and filtered and centrifuged. Chemical analysis is made on 2 ml. filtrate by adding 2 ml. of 1 per cent alcoholic resorcinol and 6 ml. 30 per cent HCl. The mixture is then heated in a water bath at 80° - 85° C. for 10 minutes, cooled and intensity of colour is read in photoelectric colorimeter at 490 mμ. The optical density observed can be read on a calibration curve and amount of fructose can be obtained.

The determination has to be carried out under anaerobic conditions and phosphate buffer depresses oxygen uptake in small narrow test tubes (Salisbury and Sharma 1957) and therefore, phosphate buffer is being used for this purpose. Bhosrekar (1975) recorded 1.4 ± 0.16 fluctolytic index for buffalo semen. Bhosrekar et al (1984) did not record any significent breed differences ordifferences due to frequency of collection for Holsten Fresian and Jersey semen. Seasonal variations in fructolysis has been reported, similarly positive correlation have been recorded between fructolysis and sperm concentration (Nakabayashi and salisbury 1956; Sengupta et al 1963; Sinha et. al.1966; Paul et. al. 1966).

Doicheva (1980) observed non significant effect of seasons on initial fructose content, however Chaudhary and Sadhu (1983) reported that initial fructose content is significantly affected by seasons, being lowest in summer.

Resazurin reduction test : Resazurin, like methylene blue is an acceptor of hydrogen and is reduced in the process. The reduction of resazurin results in a series of colour changes; in solution it is blue. When oxidised, it changes through purple to pink resazurin and finally white hydro-resazurin, during reduction. The reduction of hydro-resazurin is reversible. Erb and Ehlers (1950) have used the reduction of this dye as a measure of metabolic activity in place of methylene blue.

11 mg. of resazurin is dissolved in 200 ml. of distilled water. 0.1 ml. of this solution is added to 6.2 ml of undiluted semen and mixed in a 10 by 75 mm test tube. The mixture is then covered

with a layer of mineral oil and incubated at 40° C. Two end points may be recorded, the pink and the white. The pink end point occurs in as less time as 1 minute with better semen samples; the white end point requires three or four times as long or longer. The time to each end point is highly negatively correlated with sperm cell concentration and motility. The higher the concentration and motility, the shorter the reduction time.

The pink end point is much more closely related to fertility of semen when the test was started with fresh semen. The test was later modified for 150 x 10^6 spermatozoa in 0.2 ml. of diluted semen. (Erb and Flerchinger 1952). This resulted in elimination of the problem of varying cellular concentration.

Glutamic oxaloacetic transaminase activity (GOT) :

There is no method to predict the fertilizing capacity of semen. Motility under microscope is the most widely used technique for assessing the quality of semen, but with the advent of deep frozen semen, the recovery rates though present after thawing has resulted in either very low fertility or no fertility at all. This may be due to damage of spermatozoa during the process of freezing. To record the extent of damage occurred to spermatozoa, there is no satisfactory method except to depend upon biochemical test to estimate the presence of enzymes (limited only to cells), in seminal plasma after freezing and thawing. One such enzyme is Glutamic oxaloacetic transaminase.

This enzyme is purely cellular and is not found in seminal plasma (Pace and Graham, 1970). The presence of this enzyme in plasma indicates the damage to spermatozoa. Aspartate Aminotransferase is an intracellular enzyme of spermatozoa and its leaching into the seminal plasma may indicate damage to the plasma membrane of the sperm, and the assay of the activities of these enzymes has been used for evaluation of the quality of semen. (Flipse, 1960; Crabo et al 1971; Rao and Pandey 1977, Jani et al 1983, Bhosrekar 1974, Dhami (1986) and Bhosrekar et al 1988).

Graham and coworkers (1972) studied the effect of some buffers for freezing and storage of spermatozoa by estimating GOT activity. Crabo, et al (1972) studied this as regards boar semen. Bower, et. al. (1973) studied the effect of addition of glycerol on release of GOT in extra cellular fluid and they found that addition of glycerol always increased the GOT activity in extracellular fluid indicating thereby damage to spermatozoa.

For estimating GOT, Sigma - Frankel units are used and the method also was based on Reitman and Frankel (1957) 1 ml. of sigma prepared substrate is added to a test tube and placed at 37° C in a water bath. To this 0.2 ml. of extra cellular fluid from diluted sample is added and kept in water bath for exactly one hour. After 1 hour 1 ml. of sigma colour reagent is added to stop the enzyme activity and start colour reaction. The sample is then left at room temperature for 20 minutes. 10 ml. of 0.4 N NaOH is then added and mixed by inversion using rubber stopper. After 5 minutes the colour

is read in double cell colorimeter with green filter. Every estimate is accompanied by a blank using 1 ml. sigma substrate, 0.2 ml distilled water, 1 ml colour reagent and 10 ml 0.4 N NaOH. Corrections are made from readings of blank every time before calculations.

Alkaline and Acid Phosphatase :

Semen phosphatases play an important role in dephosphorylation during sperm metabolism. Alkaline and Acid phosphatases in semen reflect the functional state of accessory sex glands and metabolic activity of spermatozoa. Zrerava and Chuhrly (1971) reported increase in the conception rates in cows following inseminations with semen containing increasing order of Alkaline phosphatase.

According to Ibrahim et al (1985) though epididymis represents a main source of Alkaline and Acid phosphatase in semen, Ampullae share a great part in contributing these elements. At least some might be the results of leakage from sperm cells (Mahmoud et al 1986). Bhosrekar, 1974 reported drop in Alkaline phosphatase on freezing of the semen. While Dhami and Kodagali (1988) reported increase of these enzyme activity on freezing semen in the extracellular fluid. They also found significant effect of extender on the leakage of these enzymes. Incubation method of Bodansky (1932) described by Oser (1965) for alkaline phosphatase and method of King and Armstrong (1934) can be used for Acid phosphatese estimation.

Hyaluronidase activity :

Estimation of hyaluronidase has assumed a great importance in view of its place in spermatozoan system. This enzyme is present in acrosomal system of spermatozoa and nowhere else. Since integrity of acrosome is directly involved in fertilizing capacity of spermatozoa. This enzyme has assumed a great importance in estimating fertilizing capacity of semen. If there is damage to the acrosome it is presumed that this enzyme will leak out in extracellular fluid and presence of hyaluronidase activity in extracellular fluid will indicate acrosomal damage.

The semen is centrifuged on dextran raffinose layer at 12000 g for 10 minutes and plasma is separated. Only 0.5 ml is taken from the supernatant so that there is no mixing of dextranraffinose with plasma. A control for total hyaluronidase content was also run by taking a fresh ejaculated and diluted semen without centrifuging and after freezing and thawing five times.

Sample of 0.3 ml is incubated with 0.2 ml hyaluronic acid (Sigma chemicals, London) in sodium formate formic acid buffer (pH 3.8) containing 0.375 M. sodium chloride at 37° C. for 30 minutes. The reaction was stopped by addition of 0.1 ml of potassium tetraborate buffer (0.8 M pH 8.9) and the sample is placed in boiling water bath for 7 minutes. Enzyme blank sample are those to which potassium tetraborate buffer was added before incubation. After cooling, 3.0 ml of D.M.A.B. reagent (10 g diamethyl aminobenzaldehyde from sigma in 87.5 ml. glacial acetic acid) is added and

thoroughly mixed. The colour was developed for seven minutes at 37° C before the samples are cooled and the optical density is determined at 585mμ in spectrophotometer. N-acetyl glucosamine released per hour/ml by the action of hyaluronidase on hyaluronic acid is calculated by referring to standard curve prepared with N-acetyl glucosamine/ml. The hyaluronidase activity present in seminal plasma is expressed as a percentage of total activity measured in semen sample frozen and thawed 5 times. The purpose of layering on dextranraffinose was to ensure minimum breakage of spermatozoa on centrifugation. The method is by Barrett (1972).Modified by Foulkes and Watson (1974).

Kaker and Anand (1984) estimated leakage of acrosomal enzymes due to freeze preservation using different diluents. They found positive correlation between enzyme leakage and acrosomal damage. Ganguli and Kaker (1980) reported 3.34 fold higher leakage of hyaluronidase in seminal plasma when semen was treated by sonication than by freezing and thawing. They did not find any effect of diluent in release of hyaluronidase in seminal plasma. Chinnaiya and Ganguli (1982) estimated hyaluronidase in buffalo semen.They reported that, the hyaluronidase activity increased in extra cellular fluid on freezing and thawing as compared to before freezing. Dhanda et al (1981) studied sorbitol dehydrogenase and hyaluronidase activity in buffalo semen. They reported marked variation amongst individuals. According to them motility of sperm is inversely related to hyaluronidase activity. EL Danasouri (1988) observed loss of acrosomal enzymes acrosin and hyaluronidase from spermatozoa after thawing at different temperatures

and time. The highest acrosome activity remaining in the spermatozoa and the lowest hyaluronidase activity in the media were observed after thawing at 70° C. Antonynk and Bezlyerdnikov (1983) have used hyaluronidase enzyme in different proportion in diluent for diluting boar semen. They could not find any significant difference on conception rate in sows inseminated with diluted semen having hyaluronidase as compared to controls.

Morton (1975) studying by immuno cyto chemical methods in ram spermatozoa reported localization of hyaluronidase and acrosin in acrosomal region of spermatozoa. Hyaluronidase staining was denser at periphery of sperm head whereas acrosin staining was denser in the equitorial region and appeared to be bound to the inner acrosomal membrane. Flechon and Dubois (1974) used similar technique for bull, ram, boar and rabbit sperm and observed localization of hyaluronidase in the anterior part of the acrosome.

Physical methods of evaluation

* **Impedance change frequency (ICF)** : Rothschild (1948) devised a method of objective assessment. This depends upon the frequency of changes in resistance to the passage of electric current through the sample of semen. However, this test is applicable only to fresh undiluted semen where waves can be seen (Rothschild 1950 a). Cummings (1954) has studied relationship of impedance change frequency and conception rates with over 35000 first inseminations and found it to be of value in assessing semen quality. Bishop et al (1959) also reported direct significant results of their survey.

*** Filtration through sephadex :**

The prediction of fertility by laboratory assay is a major problem in artificial insemination. Centres producing frozen bull semen generally use sperm motility as the sole criterion for semen quality. Graham et al (1976) proposed a rapid and objective test of sperm viability utilizing electronic counting of diluted bull spermatozoa before and after filtration through sephadex G-15. Sperm motility and some membrane characteristics appear to be factors influencing the trapping of bull, ram, boar spermatozoa in filter. (Graham et al 1976, Graham et al, 1978; Fayami et al. 1979, Heuer et al 1983).

A slurry of sephadex G.15 (Pharmacia fine chemicals uppsala, Sweden) was allowed to swell for a minimum of 3 hrs. at room temperature. Disposable plastic syringe (2-5 or 3 ml) are fitted with loosely rolled ball of glasswool (Phyrex brand wool, Fiber glass, R-corning Glass works) which is compressed with the plunger so that it fitts the syringe to the 0.1 ml mark. Course strands of glass wool are avoided to obtain an evenly packed plug. Under constant stirring a 0.7 ml. of the sephadex slurry is transferred into syringe. Three ml. of 3% sodium citrate solution was added to the syringe twice and allowed to drain through the gravity to pack the gel. The filters are then stored vertically in a refrigerator and covered with sodium citrate solution until used.

Immediately before use the syringe is again filled with sodium citrate solution which is allowed to drain until it levels with the upper surface of

the gel. The effluent is discarded. The filterishung in the opening of the dry test tube. 0.1 ml of the extended semen (Pre or Post freeze) is added using an Eppendorf pipette and allowed to drain into the gel. Exactly 2.5 ml. of 3% citrate solution is then added and all the effluent fluid is saved for counting. A prefilter sample is prepared by adding the same volume of semen to 2.5 ml. of sodium citrate. One drop of 10% formaldehyde is added to immobilise the sperm. The sperm number is counted by haemocytometric method,. Those filtering through are all motile and live.

The filtration is affected by the temperature. Freeze killed buffalo spermatozoa were unable to pass the filter and there is a major difference in the ability of fresh and frozen sperm to go through the sephadex (Graham et al, 1976; Graham et.al, 1978; Fayami et. al. 1979) Caffeine did not increase the number of sperm passing the filter. Krishnamurthy et al (1983) Chandrahasan et al (1985) used glass wool filter for filtration of bovine semen and reported reduction in abnormal and dead sperm on filtration.

Objective method of measurement of motility :

Minitub (West Germany) has produced a completely computer assisted semen analysis system which has phase contrast microscope, camera and control monitor with personal computer with 640 KB CPU co-processor and digitizer with 256 grey valves terminal printer. This system will yield exact data regarding motility, concentration, velocity of cells, ratio of live/ dead cells, quantity of required diluent and straws. It has the capacity of storing entire production information.

Rothschild (1953) reported a method of recording movement of spermatozoa by photographic method. Though this method is costly and time consuming at the same time the rate of travel of spermatozoa across the field can be very accurately judged. Van Denmark (1955) was able to measure the speed of spermatozoan movement (selected cells) across the field under microscope with the help of stopwatch. Baker et al (1957) reported a modified and fairly accurate method of estimating time required to cross the measured area by spermatozoa under microscope by using Petroff Hausser Chamber. Rikmenspoel and Van Herpan (1957) used an apparatus designed to make photographic as well as photoelectric measurements in order to detect normal and abnormal types of spermatozoal movements as well as speed and number of moving cells.

Methods of evaluation based on resistance to environment

* **High temperature viability test** : Ludwick et al (1948) reported a high correlation between time of incubation at 100° F required for all spermatozoa to lose their motility and conception rate. Similar results were reported by Buckner et al (1954) who estimated progressive motility after incubation for 16 and 28 hours in eggyolk citrate at 38° C. Since motility is a very subjective method of estimation of seminal quality, therefore, either complete cessation of motility or absence of progressive motility should be taken as a basis.

* **Resistance to temperature shock** : Lasley and Bogart (1943) have shown relationship between survival rates after cold shock and fertility. This wasconfirmed by Bishop et al(1954). They did this by

counting the live sperm after cold shock with the help of Nigrosin-Eosin, Stain. They concluded that greater the resistance towards cold shock better is the fertility. Cold shock resistance test was also used by Mathura group of workers to evaluate/screen the bulls in India. Bhosrekar (1975) used cold shock resistance of spermatozoa for screening the bulls for their freezibility. This gave positive relationship in few samples studied. Roy (1974) showed positive relationship between cold shock and freezibility in buffalo bull. This requires plunging of test tube containing semen in ice water for 10 minutes. The number of surviving cells are determined by deferential staining. Tuli et.al. (1985) recently estimated 'GOT' in samples of buffalo semen before and after cold shock. This test can be utilised for selection of medium for deep freezing of semen. Darin Benneta and white (1974) reported loss of phospholipids, phosphoglycerides, choline, plasmologen phosphatidyl choline from sperm cells on cold shock.

Resistance to sodium chloride and dilution effect

The measurement of deleterious effect of a 1 per cent solution of sodium chloride on spermatozoal survival as reported by Milovanov (1934) was described in detail by Anderson (1945). Subsequently Chang et al (1949) and Emmens and Swyer (1948) using rabbit spermatozoa, showed that a similar effect could be obtained with chloride free diluents.

Milovanov (1934) recommended dilution of a measured volume of semen with 1 per cent NaCl solution until progressive motility of spermatozoa ceased. The amount of 1 per cent NaCl required to cease the motility reflects the resistance of spermatozoa. Resistance (R) is V/vl where V is the volume of 1 per cent Nacl and vl is the volume of semen sample.

CHAPTER VIII

PRESERVATION OF SEMEN

Diluents for preservation

The primary considerations for a satisfactory diluent should be isotonicity with sperm cells, a good buffering capacity and non toxicity with correct balance of electrolytes and non-electrolytes with a correct pH. A diluent should provide a substrate to support the reduced metabolism of stored spermatozoa and it should not have detrimental or inhibitory effect of antibiotics, which are now accepted as essential in semen diluents. The diluent should be easily prepared and should be cheap. It should permit the microscopic examination of spermatozoa in a clear field and not render difficult the cleaning of glassware and other semen containers. It should contain lipoproteins and/ or lecithin to protect the sperm against cold shock.

Milk and Milk based diluents :

Milk as a semen diluent has been reported by number of workers. The first published report of milk as a diluent appeared as early as 1940 by Phillips and Lardy. Jacquet (1951) recommended use of canned skim milk since he obtained 10 to 15 per cent higher conception rate by its use as compared to Egg Yolk citrate. In 1952 Jacquet and Cassou recommended use of skim milk powder because of variation in quality of canned products. They further suggested to use 10 per cent reconstituted skim milk with 10 per cent egg yolk together with antibiotics. Almquist (1951)

used heated and cooled skim milk as a diluent. He boiled the milk in closed vessel for 10 minutes. Sanfile (1953) reported 4.2 per cent higher conception rate with heated and cooled skim milk diluent as compared to egg-yolk citrate.

Thacker and Almquist (1953) showed that spermicidal action of unheated milk is associated with albumin containing fraction of milk. They postulated that the inhibitory factor gets destroyed on heating to 92°C for 10 minutes.

Flipse et. al. (1954) indicated that the toxic factor present in unheated milk was probably "Lactenin" an antistreptococcal agent normally present in milk in albumin fraction and could be reliably inactivated by controlled heating.

Boyd et. al. (1954) and Johnson et. al. (1955) demonstrated that the toxic factor in unheated milk could also be inactivated by the addition of sulphahydryl compounds. These authors also confirmed that 'Lactenin' a major spermicidal factor in fresh milk could be inactivated by sulphahydryl groups released through denaturation of lactoglobulins by heating or by directly adding to milk as reduced glutathione or cysteine hydrochloride. Saacke et al (1956) preferred heating over chemical treatment of milk. For all practical purposes a heat treatment of milk at 92°C for 10 minutes in water bath could be an ideal.

Homogenised milk, skim milk or skim milk reconstituted from skim milk powder can be used alone or in combination with egg yolk as a diluent for bovine semen. Milk glycerol diluent has been reported by

Williams et al (1957), and this diluent can keep the semen viable upto 3 to 4 days. O'Conner and Smith (1959) obtained 4.7 per cent higher conception rate with skim milk glycerol over skim milk alone.

Jones (1965, 1968 and 1970) used skim milk preparations for preservation of ram and bull semen and for freezing the semen. Meding (1964) used homogenised sterilized milk, homogenised milk and homogenised sterilized cream for dilution of bull semen. The non-return rates for first service averaged 65.2, 65.0 and 65.4 per cent respectively. The differences between diluents are non-significant.

White (1964) used reconstituted milk from skim milk powder and egg yolk for freezing bull semen and reported higher conception rate with this. Tomar et al (1964) used skim milk diluent for buffalo semen. Veeramani Ayyar (1952) Joshi et al (1967) have used whole milk for dilution of buffalo semen and preservation at 5° to 70 °.C Grewal et al (1969) used Milk glycine with yolk citrate for buffalo semen with advantage. Tomar and Desai (1961) used skim milk and various additives with skim milk as diluents for buffalo semen. Kale (1963) used milk-glucose bicarbonate as a diluent for buffalo semen and found it to be better than whole milk alone or glucose milk yolk, milk egg yolk or milk egg yolk citrate. Joshi (1967) tried cow, buffalo and goat milk for preservation of buffalo semen and found cow's milk to give better motility for longer duration. Guesinov et al (1969) used buffalo milk with egg yolk as a diluent for buffalo semen. Bhosrekar (1975) tried buffalo milk, buffalo skim milk, cow milk, cow skim milk and goat milk and goat skim milk for preservation of buffalo semen and found that cows whole milk as well as cow's skim milk

had higher preservation capacity than milk or skim milk from other two species. He further developed a diluent from cow's skim milk for preservation of buffalo semen by preparing whey out of it with the help of citric acid. He also used this for preservation of buck's semen with adjustment in pH. Buffalo required 6.8 pH. while goat semen required 7.4 pH.

Egg yolk based diluents :

The wide spread development of artificial insemination was largely made possible by the work of Lardy and Phillips (1939) who have shown the protective action of egg yolk against cold shock. The active principle in egg yolk responsible for this is now thought to be lecithin or a similar phospholipid occurring either free or in combined form with protein (Black Shaw, 1954; Mayer and Lasley, 1945; Kampschmidt et al 1953 a). Recently Bomstein and Steberi (1959) claimed that crude lecithin had a greater protective action than purified lecithin.

In addition to its protective action against cold shock it provides certain substrate which will support spermatozoal metabolism. Its glucose content can be utilized by spermatozoa. (Van Tienhoven et al 1952). Very recently Shannon (1973) reported that egg yolk enhances the production of toxicity from dead spermatozoa (amino acid oxidase); Japanese workers have reported the coagulation of egg yolk by buck semen. Buck semen has an enzyme called "phospholipase" which hydrolises egg yolk lecithin producing toxic factors for buck sperm. This enzyme comes from cowpers gland of buck. Basu and Berry (1948) reported that Turkey egg yolk is as effective as hen's egg yolk in storage of spermatozoa.

Egg yolk phosphate diluent : The original diluent developed by Lardy and Phillips (1939) contained equal volumes of egg yolk and a phosphate buffer (2.g. Na_2 HPO_4 12 H_2O, 0.2 g KH_2 PO_4 in 100 ml. distilled water). The diluent had one serious disadvantage in that the diluted semen had lot of fat globules which obliterated the vision under microscope and clear motility could not be seen. However, Phosphate buffer had a depressant effect on oxygen uptake by spermatozoa and has high buffering capacity and therefore, phosphate buffer can preserve the semen well under aerobic conditions. But under higher dilution rates with yolk phosphate diluents there is depressant effect on the motility of spermatozoa possibly due to interaction between yolk and phosphate ions (Salisbury, 1957).

Yolk citrate diluent : This was described by Salisbury et al (1941). The advantage with this diluent is that it gives clear field and permits microscopic assessment of motility. It has no adverse effect on the sperm metabolism. Earlier equal volumes of egg yolk and 2.9% or 3.6% sodium citrate dihydrate solution were being used, but with the recognition of economy and adverse effect of egg yolk under high dilution rates, the percentage has come down to as less as 20 to 15 per cent. So 20% of egg yolk with 80% of sodium citrate dihydrate solution of 2.9% is generally practised and is found most suitable (Almquist, 1951 a, Holt, 1952 c, Stewart, 1950). It is always good to adjust the pH to 6.8 before addition of egg yolk. No adverse effect was observed on preparation of diluent from stored sodium citrate solution upto one month (Hurst, 1953).

Modifications of egg yolk diluents : Van Demark and Sharma (1957) described a possible method of storage at room temperature (I.V.T.) with 20 g sodium citrate dihydrate, 2.1 g sodium bicarbonate, 0.4 g Kcl_2 3.0 g glucose and 3.0 g sulphanilamide in one litre of distilled water; after gassing with CO_2 until the pH had fallen to 6.3, 1000 µg. streptomycin and 1000 I.U. penicillin per ml were added together with egg yolk. The final concentration of yolk being 10%. The semen diluted in this diluent was stored in 1 ml ampoules in dark at room temperature. 75% Conception rate was reported from 111 inseminations carried out with semen stored at room temperature for 7 days. VanDemark and Bartlett (1958) found it necessary to modify by providing more sugar (glucose) and egg yolk and addition of catalase for storage at ambient temperature to obtain best spermatozoal survival rate. McFee (1958) reported satisfactory conception rate with semen stored in IVT diluent for three days. Similar results were also reported by Beljakov (1959).

Kampschmidt et al (1951) and (1953 b) used a diluent with one volume of egg yolk to a five volumes of a mixture containing 1 part of 1.3% sodium bicarbonate solution and 4 parts of 5% glucose solution. The motility of spermatozoa was found to be excellent in this diluent (Smith, 1959). Gokhale (1958) used this for buffalo semen and obtained very good results on survival of buffalo spermatozoa. Foote (1958) investigated the use of semen in a diluent containing egg yolk, sodium citrate, potassium chloride, glucose and glycine (referred to as C.U.E.) and found this to be as effective as yolk citrate over a 2 days storage.

Since it has many components and each requires careful weighing and adding and therefore, it is not

suitable for routine use. Sergin (1956) claimed addition of fructose to yolk citrate improves viability of spermatozoa though it has not been shown clearly to improve conception rate.

Mahajan and Sharma (1963) concluded that yolk glucose bicarbonate was significantly superior to other diluents for buffalo semen. C.U.E. dilutor was used by Clamohy and Palad (1961), Joshi et al (1968); Sengupta and Roy (1968) for buffalo semen and found it to be most satisfactory. Srivastava and Raza Nasir (1968) used glucose yolk bicarbonate diluent for buffalo semen and found it better, because of alkalinity the diluent has given higher livability was their explanation in favour of this diluent.

Addition of chelating agents like EDTA and DEDTC or cysteine hydrochloride or reduced ghutathione to yolk or milk based diluents showed beneficial results. Amongst the additives cysteine hydrochloride was found to give consistent significant beneficial effect from 4 to 120 hours of storage (Sengupta et al 1969).

Joshi, et al (1968) used different amino acids as additives and found 4% glycine with 25% egg yolk to give the best results.

0.26 M Tris with pH adjusted to 6.4 by citric acid 0.5 to 2.0% glucose with egg yolk gave definitely higher sperm motility over several days of storage for buffalo semen. Sengupta and Choubey (1972) Yaseen (1971) used 0.25 M Tris and 7.0% glycerol with 20% egg yolk for preservation of buffalo sperm and found it most suitable.

Yassen and Kamash (1972) in another experiment used glycine yolk extender for buffalo semen with good success. They used 1.7% glycine, 7.0% glycerol, 1.0% fructose and 20% egg yolk. However, they found it significantly inferior to Tris yolk.

Chieffi and Masotti (1956 b) reported 3 parts of Tomato juice and 1 part egg yolk to be satisfactory diluent for semen. Singh and his coworkers (1968) have done lots of experiments with tomato juice for diluting ram and bull semen and found success. Roy and Bishop (1954) reported that 4% of glycine solution in distilled water with egg yolk in equal volume gave beneficial effect on survival of spermatozoa at 5° C as compared to yolk citrate and yolk phosphate.

Rakes and Stallcup (1956) Baier et al (1957) and Saha and Singh (1958) have reported beneficial effect of glycine egg yolk on sperm survival at 5° C while Strom (1956) found no difference in fertility in split sample trial involving 3000 inseminations.

Adler and Rasbech (1956 c) obtained ô6.0 and 66.2 per cent 60-90 days N.R. rate on use of semen diluted in glycine-fructose-egg yolk and egg yolk-citrate on 8800 inseminations respectively. There is no evidence of improvement in fertility with inclusion of glycine in the diluent. Rakes and Stallcup (1950) found less buffering capacity with the glycine egg yolk diluent.

Coconut milk extender has been first investigated by Norman et al (1958). (Equal volumes of coconut milk and 43.2% sodium citrate solution plus 100 mg calcium carbonate, 80 mg of penicillin and 90 mg of streptomycin). Later Norman and his coworkers brought improvements and used this diluent for room

temperature preservation with additions of catalase polymixine and mycostatin. Norman et al (1968) used this diluent for buffalo semen and could preserve buffalo semen for seven days at room temperature with good motility.

Commercial diluents : The exact composition is never disclosed. In Europe such diluents are available for semen dilution. eg. Spv 161 was found as good as egg yolk citrate by 'Kust and Jurgens (1951.) Spermasol Plus 20% egg yolk gave best results (Hoelzer; 1951, Kobert, 1952, Bonfert 1953; and Bonfert 1956 a). 'Seminan' another diluent used by Nishikawa et al (1955) contained 25% egg yolk 1.6% sodium citrate, 0.11% potassium citrate, 0.15% sodium phosphate 0.47% glucose, 0.1% hemosulphamine and 0.05% sodium sulphamesadin in distilled water. Lots of reports are available about laciphos, as a diluent for bovine semen (Cassou 1968). It has been used all over Europe and America as a diluent for bovine semen. Along with egg yolk and glycerol it is used for diluting bovine semen for deep freezing.

Diluters for Deep freezing : Milk with 10% glycerol was used by Dunn and Hafs (1953) O'Dell and Almquist used skim milk heated to 92° C for 10 minutes with glycerol as a diluent for freezing semen.

Number of workers have used egg yolk citrate with glycerol as diluent for freezing semen with or without fructose. Different percentage of glycerol content in diluent was considered for suitability in freezing bovine semen. All results were based on recovery rates or fertility results on 60-90 days non-return, with egg yolk citrate diluent 7% glycerol was found suitable.

Nagase and Graham (1964) used sugar diluent for diluting and freezing semen in pellet form. They used 10 per cent lactose with 4% glycerol. They also used number of other sugars like raffinose, glucose, arabinose or combinations of these.

Addition of glycerol fraction is preferred at 4° to 5° C. (Miller and VanDemark 1952) but no adverse effect on post thaw recovery rates was reported on addition of glycerol fraction at room temperature or little below room temperature. (Polge and Jacobson 1959).

Graham and Coworkers (1972) used Zwitter ionic buffers titrated with bases. They used Tris at pH 7.0 and reported higher motility of spermatozoa in this diluent. Simmet (1975) used Tris glycerol diluent prepared in concentrated form (Triladyl) to be diluted later with egg yolk and distilled water in proportion of 25:25:75 for diluting bovine semen at room temperature and directly filling and sealing, and then freezing after equilibration at 5°C This has given improved fertility over Cassou's method of freezing. Anderson (1974) found no significant differences in fertility rates of Cassou straws and Simmet's landshut Minitubes.

Roussal et al (1972) used Mono sodium glutamate solution for freezing bull spermatozoa. Lopatko and Tyupina (1972) used raffinose glycerol egg yolk as most suitable medium for freezing bull semen.

Pavitran et al (1972) reported milk egg yolk sodium citrate lactose and glycerol diluent for freezing of buffalo semen. Recently Roy et al (1974) have published reports about freezing of buffalo semen in

sodabicarb-glucose-eggyolk diluent with 60% recovery rate, while Bhosrekar (1974) has evolved a new diluent citric acid whey for preservation of buffalo semen at 4^0 to 5^0 as well as ultra low temperatures by deep-freezing technology. He used the deep frozen buffalo semen on 500 buffaloes from Buffalo Farm (NDRI) and reported 63% conception rate on first service with straws and 58.33% with ampoules.

Some common diluents which could be used for freezing cattle and buffalo semen are as follows : These are prepared in double glass distilled water.

Diluent I	Diluent II
Sodium Citrate 2.9% W/V Fructose 2.0 W/V Egg Yolk 10.0 V/V Penicillin Sodium 500 lu/ml Streptomycin 500 mcg/ml.	All the rest of constituents plus 16%glycerol
II. Sodium Bicarbonate 1.3% 1 part Glucose 5% 4 parts Egg yolk 1 part	All the rest of constituents as diluent I plus 14% glycerol
III. Skim milk heated to 92^0 C for 10 minutes & Cooled 80 parts Egg yolk 20 parts	All the rest as diluent I plus 20% glycerol

Citric Acid Whey : Ready formula mix-in 100 ml. distilled water filter through cotton in another clean beaker, adjust the pH of filtrate to 6.8 exactly with freshly prepared 10% NaOH, add penicillin and streptomycin at 1000 IU per ml and 1.5 mg per ml.

Divide in equal volumes as Diluent I and II. To diluent I add 3% glycerol and diluent II add 11% glycerol.

Laciphos - 123 prescribed with 10% egg yolk-one bag of laciphos gives 550 ml of the dilutor.

Tris Diluent.

Formula 1	Formula 2
(a) Tris 3.87 g	TRIS (Hydroxy methyl)
(b) Fructose 1.27 g	amino methane 12.1g
(c) Citric acid 1.73 g	Citric Acid 6.8g
(d) Dist. water 99.13 ml	Fructose 5.0g
mix a, b, c, d well	Glycerol 320 ml.
	Sterile distilled water 368 ml.

Standard solution	74.0 ml
Egg yolk	20.0 ml
Glycerol	6.0 ml
Dihydrostreptomycin	1.0g/litre

pH adjusted to 6.8 with citric acid or NaOH

Egg Yolk citrate diluent

DILUENT I	
Sodium citrate 2.9%	75 ml
Egg yolk	25 ml
Fructose	2.5 g

DILUENT II	
Sodium citrate 2.9%	75 ml.
Egg yolk	25 ml.
Glycerol	14 ml.

Techniques of preservation

Preservation as liquid semen at 5-7° C (Refrigerator)

Semen after collection is evaluated for its quality and if quality is satisfactory is diluted in suitable extender either in egg yolk citrate, milk or skim milk egg yolk or soda bicarb glucose egg yolk in suitable storage tubes. Through the hole of a rubber stoper a glass tube with rubber adapter is put inside the tube. The tube of diluted semen is then kept in a plastic beaker containing water at 35° C (same as water bath in which semen was kept). The beaker is then kept in refrigerator for preservation. The semen thus preserved maintains good motility upto 72 hours and in some cases upto one week. The fertility is also good upto 3 days.

The inseminations are done with the help of glass syringe and a glass pipette with a rubber connector. The glass syringe is attached to a rubber adoptor in a semen storage tube, the semen is sucked. The syringe is then attached to a glass pipette through a rubber junction. The pipette is introduced in vagina by a inseminator while one of his hands in rectum holding cervix and directing pipette into uterus. The semen is then deposited at 3/4 cervix (Recto vaginal method).

Deep-freezing : Indefinite preservation of living substances has long been a goal of research. The main idea of deep freezing was to arrest the processes of life. Attempts to preserve spermatozoa by deep freezing have been recorded as early as the work of Spallanzani (1803) Devenport (1897) had reported

the capacity of human spermatozoa to withstand freezing at -17° C but little attention was paid to this until Jahnel in 1938 noted a survival of a portion of human spermatozoa when frozen in glass tubes and held for long periods at -79° C in solid CO_2. Luyet cited by Hoglund and Pincus (1942) reported the crystallization of protoplasmic systems at -30° C to -40° C and therefore, the survival was promoted on rapid freezing through this range. Hoglund and Pincus (1942) observed that human spermatozoa with stood vetrification better and that a higher percentage revived than the spermatozoa of rats, mouse, guinea-pig, rabbit and bull. Luyet and Hodapp (1939) partially dehydrated frog spermatozoa by dipping in one molar sucrose solution and freezing in thin films on a mica cover glass by plunging in liquid air. This procedure minimised the ice crystals from water with in the cells and instead the spermatozoa became vitreous. Shaffner et al (1941) could find no damage to fowl spermatozoa on freezing with fructose and storing at -76° C for varying lengths of time. Rostand (1946) reported that glycerol in final concentration of 20% protected frog spermatozoa frozen to -6° C and semen not found solidified at that temperature. The chance discovery of Polge, Smith and Parkes (1949) not knowing the work of Rostand showed that addition of 15 to 20 per cent glycerol protected fowl spermatozoa on deep freezing and good motility was demonstrated by them after thawing. This work of Polge and Coworkers gave an impetus for further research in this line. Later number of workers have carried out research regarding the percentage of glycerol, equilibration time required, packing system etc., for different species of live stock.

Physiology of deep freezing : The spermatozoa are subjected to two hazards during freezing, one being the risk of temperature shock below -12^0 and the other physical changes in the medium surrounding the spermatozoa. The latter effect is due to excessive concentrations of electrolytes when water in the medium was changed to ice.

* Sperm are surrounded by a semipermeable membrane. When sperms are suspended in a medium, both extra cellular and intracellular crystals are formed on freezing. As the crystals consists of water the concentration of electrolytes will consequently increase during the crystallization period. The concentration in the medium may be so high that the lipoprotein membrane of the sperm is damaged and its permeability is changed to such an extent that the sperm dies or looses its fertilizing ability. During the thawing procedures the water crystals will thaw first, this phenomenon will lead to hypotonicity which seems to be even more harmful to the cells, and perhaps most of the damage to the cells takes place during thawing.

* Cold shock is characterised by irreversible changes of sperm caused probably in the first place by denaturation of the cellular proteins. Cold shock is caused by a too fast cooling rate.

* The size of the crystals depends solely on the freezing rate.When the difference in temperature is limited, (the freezing rate is slow), the formation of crystals will be slow and big crystals are formed. The formation of intracellular crystals may cause irreversible mechanical damage to the cell and thereby its death.

To reduce the harmful effect caused both by crystal formation process and by the changes in concentration of electrolytes, glycerol is added to the medium. The protective effect of glycerol has not been fully explained. It modifies, however, the crystal formation in the medium, so that mechanical damage to the sperm during crystallization process is reduced, partly because the crystals become smaller and the crystal mass more homogeneous. Glycerol also enters the sperm and partly replaces, partly binds its content of free water resulting in a sort of osmatic buffer action. To protect the sperm against cold shock egg yolk is added to the medium. Egg yolk protects the sperm against cold shock by its contents of lecithin and lipoproteins which contribute to the preservation of lipoprotein sheath.

It is very important that freezing rate is balanced in such a way, that the freezing is fast enough in order to avoid too much damage to the sperm due to crystallization and changes in the concentration of electrolytes. On the other hand it is just as important that the freezing rate is not too fast so that damage caused by cold shock will be the result.

It has been recently demonstrated that if the semen is exposed to temperature above -120° C changes in the crystal mass will take place. It is therefore very important to keep constantly the frozen semen under liquid nitrogen level till its use for A.I.

Lovelock and Polge (1954) demonstrated that glycerol prevented this electrolyte concentration from rising above the harmful level. Parkes (1956) has shown that glycerol permits sufficient slow cooling over critical temperature, thereby reducing the risk of

temperature shock, which normally would have been happened due to rapid cooling. In order to avoid exposure of spermatozoa over a long period to ultra low temperatures, glycerol is being used.

The use of glycerol, therefore, enables the freezing to be carried out slowly enough to avoid thermal shock without expensing the cells to lethal concentrations of electrolytes.

Glycerolisation procedures : Glycerol can be split in two fractions of diluents at 3% and 11% or straight can be added to diluent at 7.0%. Slow glycerolization at 5° C was recommended earlier by number of workers. (Dunn and Hafs, 1953; O'Dell and Almquist, 1954; Kinney and Van Demark, 1954; Saraff and Mixner, 1955). Graham et al. (1958); Settergren, (1962) Polge and Jakobson (1959) reported no drop in post thaw motility or fertility of frozen semen when glycerol was added at room temperature as compared to addition of glycerol at 5° C. While Blackshaw (1955), Almquist (1959), Stewart (1961) Colas (1975) preferred to add glycerol at 5° C and they claimed best results when glycerolization was done at 5° C as compared to room temperature dilution in single step.

Dilution in two steps i.e. initial dilution at room temperature and final dilution with glycerol containing diluent in 3 to 5 fractions at 5° C was advocated for obtaining best results by several workers (O'Dell and Almquist, 1954, Kinney and Van Demark, 1954); Saraff and Mixner 1955 Stewart 1961, Bhosrekar, 1975, Bandopadhyay and Roy; 1975, Pandit et al. 1977, Chinnaya et al 1979 and Bhosrekar et al. 1988).

Room temperature dilution by dilutor containing glycerol in single step has been advocated recently for dilution of cattle and buffalo semen (Simmet, 1972 Bhosrekar et al 1988; Abhi (1982) Matharoo and Singh, 1980, Chauhan et al 1981, Sengupta et al 1982; Shukija et al 1983). Several workers tried split sample method for diluting semen with 5, 7, 9, 11, 13, 15% and 20% glycerol and almost every scientist came to the conclusion that 7% glycerol is the most suitable level for freezing bull and buffalo semen Wiggin and Almquist, 1975; Bhosrekar, 1975; Becker et al, 1977. Pandit et al 1977). While some reports mentioned 5% glycerol is better than 7% or 8% glycerol is better than 7% glycerol. (Jainudeen and Santhana Dass, 1982 Jairaman et al, 1979).

Rajamanan et al (1971) held samples of semen for 0.5 hour at 35° C and found improvement in freezability based on post thaw motility. It is also our experience at BAIF that semen samples showing poor freezability if held for 5 to 10 min. at 35° C get improved in freezability and show higher post thaw motility.

Equilibration time : Equilibration time is that period of time which is allowed for glycerolated and packaged semen before freezing. This is carried out at 5° C and allows the sperm cells to become permeated for glycerol and an ionic and osmotic equilibrium is established between the sperm cells and its media. The composition of diluent, sperm concentration and freezing rate influences the optimum equilibration time (Polge, 1968).

Additions of sugars like fructose or arabinose in addition to glycerol help to reduce equilibration time. There are many conflicting reports regarding the

length of equilibration time and its effect on spermatozoal survival rates. It has been reported that equilibration as short as 1 minute could be employed for successful freezing (Jondet, 1972). The earlier recommendations to equilibrate semen for a period of 15 to 20 hours is not generally followed now. However, fall in post thaw motility or fertility could not be observed because of reduction in equilibration time to 4 to 6 hours. The reduced equilibration period is preferred because of advantages in the working schedule of the laboratories.

Polge and Rowson (1952) suggested in their first experiments the equilibration time of 18 hours. Myers (1954) found 14 hours to be optimum equilibration time while Dunn et al (1953) obtained best viability with five hours equilibration. Stewart (1961) showed better results by equilibrating semen for 5.5 hours than with 24 hours. Settergren (1962) found survival rate of frozen semen with 4 hours equilibration to be as good as 16 hours equilibration time. Martin (1966) used equilibration time of 2 to 18 hours and found equilibration of 8 hours to be better than 2 hours or 18 hours. Kalugin (1971) studied the effect of equilibration time from 0, 2, 4, 6, 8, 10 and 12 hours on recovery rate and found that 10 hours equilibration was better. Weitze (1973) found reduction in equilibration time reduced the motility. Wiggin and Almquist (1975), designed a factoral experiment for determining optimum equilibration and thawing temperature and reported that half an hour equilibration and 95° C thawing temperature for 7 seconds was most optimum from the point of view of intact acrosomes.

Enner et al (1976) showed that 2 hr equilibration and thawing at 75° C for 6 seconds resulted in good

motility of sperm as compared to 4 to 6 hrs. equilibration and thawing in ice water or in palm. Wiggin and Almquist (1975) also reported similar finding. Bhosrekar et al (1986 a) observed that either 6 hrs or 16 hrs. equilibration for diluted glycerolated exotic semen did not show any significant difference based on post thaw sperm motility while the same authors (1986 b) have reported 16 hours equilibration period to be significantly better for diluted glycerolated cross-bred bull semen. Earlier wells and Hafley (1974) reported significantly better results with longer equilibration periods. For buffalo semen 3 hrs equilibration is the most ideal (Bhosrekar 1988).

Additives to semen diluent :

Inclusion of sugar in the diluent has been reported to give better survival of semen after freezing (Emmens and Martin, 1957) Nagase and Graham (1964), Lopatko and Tyupina (1974) used sugar diluents for freezing semen in a concentrated pelleted form. Glucose, lactose, raffinose or combinations of these were used. They have not observed any statistically significant difference between the diluents in fertility trials. Jones (1970) used dimethyl suplhoxide, glycerol and reconstituted skim milk for preservation of spermatozoa. Roussel et al (1972) used monosodium glutamate solution as a freezing medium for bull spermatozoa, Lopatko and Tyupina (1972) used 8.5% raffinose and found to be the most suitable for getting optimum recoveries. Sengupta et al (1969) Abdou and EL. Guindi (1977) used additives like cysteine hydrochloride, EDTA and DEDTC or reduced glutathione with skim milk egg yolk diluent and obtained better results on survivability of buffalo spermatozoa. Glycine and or fructose was used in skim milk egg

yolk diluent by Tomar and Desai (1961) Joshi et al (1967) Sengupta and Coworkers (1969) Bhosrekar (1975) and Bhosrekar et al (1988) with beneficial results. Based on the assumption that the relatively poor freezability of buffalo spermatozoa than cattle spermatozoa could be due to an inherently fragile sperm plasma membrane in the former species, studies were undertaken by Sengupta et al (1982) Sukhija et al (1983), Sukhija, (1984) and Tuli et al (1986). They have tested the efficacy of membrane stabilizers Viz chloroquin diphosphate and Chlorpromazine hydrochloride at two concentrations (10.5 and 10.4 M). Both the additives improved freezability of buffalo spermatozoa significantly in terms of post thaw motility. The lysozomal stabilizer at (10.5 M) (50 mg/ml) was more effective. Addition of 5 to 7 mM caffiene puris was tried by number of workers with beneficial results (Hukeri, 1988; Miyamoto and Nishikawa, 1970; Doicheu and Paguignon, 1980; Vengust et al 1981; EL. Manoupy et al 1986 Gehlaut and Srivastava, 1988 and Bhosrekar et al, 1989). Foote and Arriola (1987) and Arriola and Foote (1987) reported addition of detergent to semen extendors at the rate of 0.5% (Vol./Vol) improved the post thaw motility significantly and the acrosome was better preserved. The detergent added was the mixture of sodium lauryl sulphate and Triethenolamine (ST LS). Bhosrekar et al (1989) observed improved post thaw motility in buffalo and cattle semen by addition of 0.5% of sodium and Triethenolamine lauryl sulphate (STLS) to glycerol fraction of diluent and adding this fraction in 3 to 5 portions to semen containing portion at 5^{0} C temperature. ST LS in presence of egg yolk exerted a change in the extending medium causing shift in increased permiability and reduced osmotic shock.

The effect can be seen on dilution of semen in one step at room temperature by glycerolated diluent with and without STLS. (Arriola and Foote, 1987). Testicular (and or epididymal extract (AISF) extract to the extent of 20% or 100 mg was added to diluent before freezing of semen. The conception rate reported was 63.2% as compared to control 60.8% having no extract (Volosevich, 1974, Dhingra et al, 1984).

Shannon (1968) used a nitrogen saturated diluent "Caprogen" for freezing bull semen. According to him the number of sperm per insemination dose could be reduced far below the optimum level by use of this diluent without adversely affecting the conception rate.

Foote (1976) used a "Capacitase" a product combining ß-amylase and ß. Glucoronidase in whole milk-glycerol diluent. The semen so treated was frozen and used for insemination. He also used another treatment of 10 mg of Catalase with the diluent per ml. and semen so treated was frozen and used for AI simultaneously. Over 16842 inseminations were carried out with all treatments. He did not record any enhanced fertility of frozen bull semen by the treatment of ß. glucoronidase, ß. amylase or catalase as compared to control.

Stolbov and Rimanova (1984) reported that, addition of vit C (ascorbic acid). 20 mg per 100 ml of diluent gave best results of motility. EL Manoupy et al (1986) reported that addition of caffeine to buffalo semen improved motility atleast by 1 to 5 times. Singh et al (1986) used 7 mM of caffine during preservation of buffalo semen at 5° C and reported improvement in motility.

Anderson et al (1965) and Ziljcov (1966) found that capronic acid at the rate of 0.07% in egg yolk citrate buffer improved motility on storage at 4° C.

Almquist and Zaugg (1974) found combination of penicillin and neomycin in 1000 units plus1000 mg or lincomycin 150 plus spectinomycin, 300 µg as satisfactory substitute for penicillin and streptomycin. Golubeva (1970) studied effect of different doses of Penicillin and streptomycine on semen quality of bull. They reported drop in sperm motility in 60% of the samples when a dose of 12500 to 500,000 I. U. of penicillin per 100 ml of diluent was used. A dose of 50,000 I.U. streptomycin recorded motility resistance while a dose of 100,000 was almost lethal and doses of 250,000 or 500,000 were lethal to spermotozoa. Therefore, while using penicillin and streptomycin recommended doses only should be carefully added to the diluent to avoid the adverse effect.

Addition of antibiotics viz. tylosin 100 µg, gentamicin 500 µg and lincospectin 300/600 µg dissoloved in 0.02 ml of double glass distilled sterile water to each ml of neat semen before addition of diluent protected the semen against organisms like bovine mycoplasma, urea plasma, campylobacter foetus subsp venerealis and Haemophilus somos. The antibiotics are required to be added in such a way that the semen is not exposed to it for more than 5 minutes.

Non glycerol portion of the diluent also should be added similar quantities of tylosin, gentamicin and lincospectin per ml. This combination of antibiotics has not been found to affect either the quality or fertility of frozen semen (Shin et al 1988; Lorton et.al. 1988a,b).

Packaging Macpherson (1954) reported ampoules sealed with rubber stoppers for packaging but leakage of alcohol in ampoules (if rubber corks are not good fitting) happened and therefore, hermetically sealing of glass ampoules was adopted. Van Demark and Kinney (1954) found great difficulty in preventing leakage in such ampoules on freezing in alcohol - Co_2 ice bath. Dunn et al (1954) used polythene bulbs for freezing semen, but Musgrave and Heath (1957) found low fertility with semen frozen in plastic bulbs as compared to glass ampoules. Graham and Erickson (1959) have reported low conception rate with semen frozen in plastic containers as compared to glass ampoules. Erickson and Graham (1959) demonstrated higher conception rates of semen in pull sealed ampoules (hermetically sealing) than in tip sealed ampoules.

The origin of present "French Straw" is from the Sorensen's straws. When Robert cassou visited Denmark in 1948 Prof. Sorenson was already using 1.2 ml straws for insemination of liquid semen as a single dose. Prof. Sorenson used to have gelatin sealing on either side of the straw. Professor also had developed a special stainless steel gun for inseminating semen contained in such straws. Cassou in 1953 developed a special plug for these straws and established the factory for manufacture of such straws in 1955, since many countries have come forward to use these straws for semen freezing. At present whole of European continent as well as America, Canada and many of the developing countries are using cassou's French straws for packaging semen for freezing. Cassou could not overcome the difficulty faced for full automation in filling and sealing of these straws. The other end required sealing with polyvinyl

alcohol powder. Very recently Cassou (1971) has developed a fully atuomatic filling and sealing unit which does ultrasonic sealing.

The 1.2 ml polyvinyl chloride straws of Sorensen were improvised to 0.5 ml (Medium French Straws) by Cassou (1964). Cassou (1968) brought further improvement by reducing the volume to 0.25 ml by reducing the diameter of the straws called 'Mini Straws'. Simmet (1972) simultaneously thought of automation in filling and sealing of straws in order to minimise warming of straws by handling, at the same time automation will be more hygeinic and sanitory.

It has been noticed during freezing experiments that the medium straws of Cassou had better surface area and weight combination and semen frozen in it had higher fertility rate as compared to ministraws which had same surface area but less weight (Simmet, 1975) (Personal communication) on the contrary the plug in the french Straw occupied lot of space making the final volume in medium straw to be 0.4 ml. Also some part of the semen is wasted for wetting the plug. If plug can be replaced by some thing which will occupy less space at the same time seal effectively, it will also solve the problem of automation and so Simmet (1972) has used steel and glass balls to seal the french medium straw without plug i.e. 90 mm and found it most suitable for freezing semen. The balls also sealed straw perfectly well hence no leakage and no trouble in insemination. He further reduced the size of minitube to 65 mm to contain 0.3 ml of semen volume. This method is known as "Landshut" method of packaging and freezing semen. It is fully automatic. The straws are 65 mm being (half of Cassou medium straws) and are sealed by steel and

glass balls. The semen packaged in it is 0.28 ml. These straws are known as miniutubes. The printing is very fast and clean. The filling and sealing machine fills and seals 4000 straws in 1 hour. The sheath for insemination required special system by which the ball and straw could be retained in the sheath allowing only semen to enter the uterus.

The package size may influence the conception rate because of the better handling possibilities and not because of the volume introduced into the uterus. Hafs et al (1970) found 2.7% points drop in non return rate when 0.5 ml. frozen semen was used in comparison to 0.9 ml. when both were packed in 1 ml ampoule with equal number of spermatozoa. Cassou (1967) recorded higher fertility in 0.5 ml. French straws as compared to 1.2 ml. French straws. Cassou (1968) further reduced the package size to 0.25 ml. and did not record consistent fertility merit over 0.5 ml straw. A variation of 0.6 to + 1.2 percent points is recorded in respect of 0.25 ml. straws as compared to 0.5 ml. straws. (Cassou, 1968; Cassou, 1972; Macpherson et al 1974.). Jondet (1972) has not recorded any deleterious effect of reducing the volume of insemination dose to 0.25 ml. Considerable advantages in using 0.25 ml. straws are because of increase in storage space without decreasing fertility of the semen.

Indo-Swiss project at Matupati using both packaging material (i.e. 0.5 ml and 0.25 ml straws) did not record any significant difference in fertility.

New German automatic machine for semen filling and sealing in minitubes has been introduced which fills the semen by aspiration technique avoiding losses

of semen by leakage (Simmet, 1988). No syringe is required and only filling nozzle and hose and sinker has to be replaced after each bull's semen.

Similarly automation has been brought by Cassou in filling and sealing of French Medium as well as mini straws by introducing ultra sonic sealing device in MRS 3 x 3 and 1 x 1 machines.

*** Advantages of minitubes for freezing :**

* The volume of semen is not much changed from that of French Medium Straws but has increased the productivity to double. The size of goblets is reduced and more handy.

* Fully automatic, more hygeinic, through use of ball sealing and less direct handling of straws.

* Double the number of straws can be accommodated in the same space as compared to French Medium straws. So the capacity is doubled thus more economic.

Deep freezing methods

Deep freezing can be done by two methods :

(i) Instant freezing
    (a) Pellet freezing and
    (b) Vapour freezing and
(ii) Slow freezing

Most commonly used method is Instant freezing. In Europe, America, Canada, Australia and other developed countries vapour freezing is followed while

Japan, East Germany and some part of Finland and West Germany are still using pellet freezing.

Vapour freezing : Jondet (1964) and Cassou (1966 and 1968) described the methods through their published bulletins for freezing of medium straws (0.5 ml.) The equilibrated straws after drying in cold cabinet are arranged on freezing ramps with the help of straws spreader. The ramps are then placed on grill in 250 litre liquid nitrogen container (LR 250). The grill is placed at a depth of 30 mm below the top brim of the container and liquid nitrogen level is kept at the grill. The freezing racks have the height of 4 cm and at the level of straws the vapour temperature is about -155°C to 180° C within 8 to 9 minutes the inside temperature of the straws reach -140° C. The freezing rate varies with the depth of vapour in the tank, liquid nitrogen level in relation to the straws and number of straws frozen at a time, volume of semen and type of diluent. Approximately 600 minitubes or 300 medium straws can be frozen at a time on a grill in 250 litre container. After allowing 10 minutes the straws are collected with the help of special forceps and placed in precooled goblet. The goblet is then immersed in liquid nitrogen. The temperature of liquid nitrogen is -196° C. Bulls spermatozoa can survive the temperature of liquid helium (-269° C) (Nishikawa et al (1972). Almquist and Wiggin (1973) investigating the different methods of freezing have reported that best revival rate was in single straws arranged in racks and horizontally frozen in static vapour of liquid nitrogen. Since the nitrogen gas has a low heat capacity per unit volume (1/1500 of alcohol) a small amount of heat will warm the gas a great deal. Thus the static vapour equilibrium can be greatly disturbed. In addition the low thermal conductivity of nitrogen

gas creates problem for uniform temperature through out the freezing chamber. Therefore the freezer should be kept closed for sufficient time between freezing operations to ensure that the required vapour temperature is reached and the chamber has a uniform temperature at least at the freezing level of the freezer. Opening the freezer during freezing should strictly be avoided.

Pellet Freezing : This was used by Japanes workers Nagase and Niwa (1963) using sugar solutions, 7.5% glucose, 10.5% lactose or 18.0% raffinose containing 5 to 5.5% glycerol with equilibration of 5 to 10 hours. The semen was placed in small drops of 0.5 to 0.2 ml using microsyringe. The droplets are placed for 10 minutes on dry ice then transferred into the containers and stored in liquid nitrogen. Nagase et al (1963) Nagase and Graham (1964) and Nagase and Niwa (1964) worked out the factors affecting the survival of spermatozoa after pellet freezing and the conception rate with frozen semen in pellet form. They have also worked out the thawing mixture for pelleted semen. They have reported higher fertility with pelleted semen as compared to ampoules or straws in Japan. Alder et al (1968) found 15% less of spermatozoa in use of pellets as compared to straws. Siefert and Beller (1967) developed inexpensive method of pellet freezing using liquid nitrogen instead of Co_2 ice. The pellets can be frozen on nylon thread which is put on Co_2 ice block. It will help in tagging for identification.

Disadvantages with Pellet freezing : Pellets are unpacked and partially diluted semen frozen in the form of a drop.

* Pellets do not provide uniform handling possibilities.
* Identification of pellets is difficult.
* Since it is unpacked contamination possibilities are more during processing and insemination
* Wastage of semen during insemination.
* Inter pellet transfer of spermatozoa under liquid nitrogen is possible causing difficulty in identification of parent hood.

Slow freezing : Slow freezing was carried out in alcohol - Co_2 ice bath. The temperature of alcohol Co_2 ice bath is brought to 4° C the same as that of equilibration. The equilibrated semen ampoules are then immersed in alcohol Co_2 ice bath. The temperature is recorded with the help of frozen semen thermometer (+30 to -200° C). The Co_2 ice blocks are put one by one slowly so that the temperature goes below 4° C at a definite rate. The rate is as follows :

+ 4° to - 10° at the rate of 3° C per minute
- 10° to - 20° / one minute
- 20° to - 30° / 5° C per minute
- 30° to - 40° / 10° C per minute
- 40° to - 79° / Fast

The ampoules thus frozen can be preserved in alcohol Co_2 ice bath or can be transferred to liquid nitrogen container in canisters for long term preservation. This process also can be achieved by automation using biological freezers and freezing tunnel. Where the air circulation is forced over liquid nitrogen to give controlled vapour around ampoules. The temperature is recorded by potentiometer recorder. The freezing rate can be controlled and slow freezing can be achieved by freezing in conventional Co_2 alcohol bath and forced controlled vapour freezing in biological

freezer or by freezing directly in crushed Co_2 ice (Jakobson, 1959; Bruce, 1956). No difference was recorded in quality of frozen semen.

Cassou (1987) has introduced "Digitcool" which has a computerised freezing rate and recording system. The racks can be kept in the chamber which is brought to + 5°C by liquid nitrogen vapour and the temperature can be lowered to - 140° at steady rate as desired. The chamber has a capacity to freeze more than 3000 straws at a time. Similarly M/s Planner Blomed (U.K.) have brought similar computerised freezing with recorder machine for bovine semen. (Fig. 20)

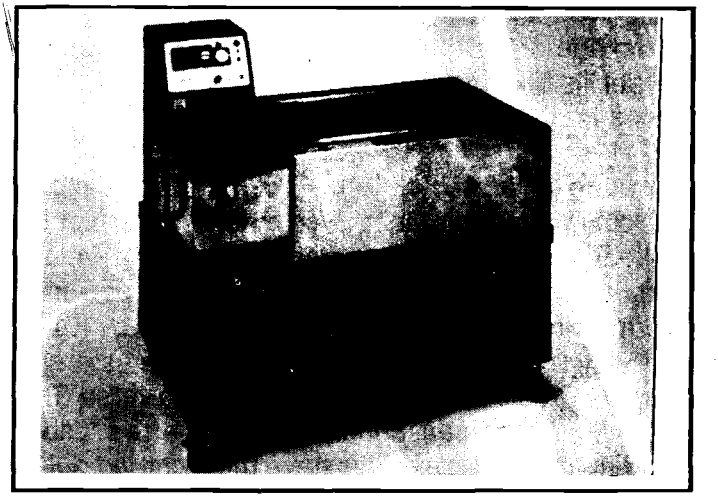


Fig. 20

Freezing rate

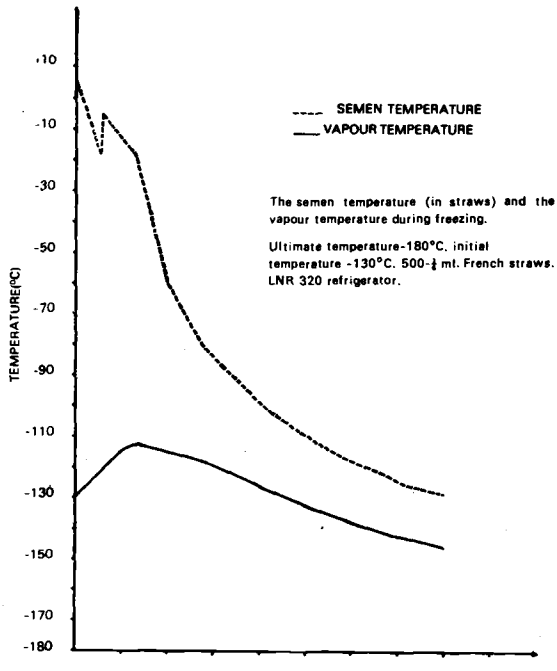
Rodriguez et al (1975) used three freezing rates 3-5 min (fast) 20 (moderate) and 40 min (slow) for cooling from + 5° to - 130° C and reported that fast freezing resulted in better post thaw motility than moderate or

slow (P <0.05). Almquist and Wiggin (1973) used 4 methods for freezing i.e. rapid forced vapour. 2) Controlled forced vapour 3) Static vapour - 170° C goblets on rack and 4) static vapour single straw on racks - 180° C. They reported maximum post thaw motility in single straw frozen in static vapour at - 180° C. Settergren (1963) used rapid method for deep freezing of bull semen and found satisfactory results. Bhandari et al (1982) used 4 speeds to go to - 140° C i.e. 10, 7, 5, and 3 minutes respectively. Post thaw motility obtained at fastest rate (3 min.) was 44.33±0.86% (P<0.01). Allen and Almquist (1981) did not record any significant difference between vertical bulk freezing in a mechanical progremmable freezer and individul horizontal freezing of straws in static vapour of LN. They, however, reported advantage of meehanical freezer over stetic vapour freezer in freezing 5 to 10 times higher number of strews in a single batch. (Fig. 21)

Handling of frozen semen : The basic principle of any cold storage is that the cold chain must not be broken. Even at frozen state the temperature fluctuation adversely affects the fertilizing power of spermatozoa. This has been shown clearly by Kalev and Zagorzki (1968) in changing the storage temperature from - 79° to - 196° C and vice versa. The deep frozen semen should be transferred under liquid nitrogen. Researches have been carried out to study the effect of exposure of straw once, twice thrice etc to atmosphere and then dipping in liquid nitrogen on the revival rates and it was reported that this practice had very adverse effect on spermatozoal survival. Almquist and Wiggin (1973) have reported that any condition which will cause rise in temperature from - 180° to - 120° C will affect the spermatozoal life

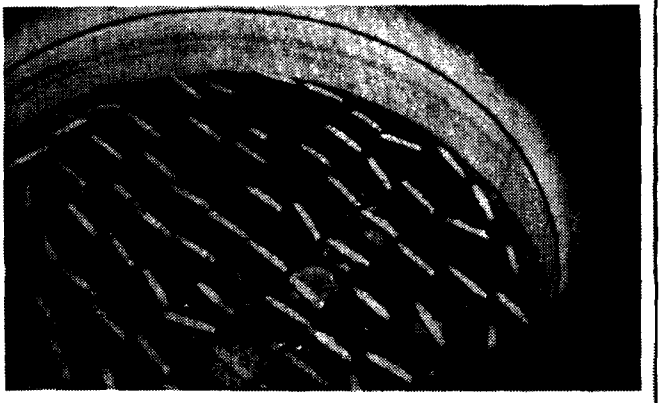
under storage. Therefore, the straws of frozen semen or ampoules of frozen semen should be transferred under liquid nitrogen to other container. This can be effectively done by taking liquid nitrogen in wide mouth container (Thermocole container) and keeping the goblets from which the straws are to be transferred and also empty goblets to which straws are required to be transferred in liquid nitrogen fixed in some gadget The straws can be picked up by long forceps (15 inches) and slowly transferred to empty goblet under liquid nitrogen surface.

Fig. 21



Method of storage : Soon after the temperature of straws reach about - 140° C the straws are collected by pre cooled forceps and transferred into pre cooled goblets. These are then immersed in liquid nitrogen and goblets are then kept in already identified conisters under liquid nitrogen. The semen should be stored for one month in the container in which it is frozen then it should be transferred to another container. This is very essential from quarantine point of view since Foot and Mouth Virus appears in semen much before the bull suffers clinically from Foot and Mouth (since incubation period is about 8 to 10 days) and it has been shown that Foot and Mouth virus as well as Infections like bovine rhinotrachaetis and Infectious pustular vaginitis virus could be preserved under liquid nitrogen along with sperms. Therefore, it will be dangerous to transfer semen immediately to long term storage container unless it is quarantined for one month otherwise there is likely hood of spreading the disease (Fig. 22).

Fig. No. 22



There are goblets available in different capacities and sizes for example bigger goblets hold 300 medium straws, small goblets 80 straws even smaller 25 straws. In polythene as well as aluminium material the goblets are available. Polythene goblets are suitable. The size of goblets vary according to size of canister. Goblets to hold 200 straws are suitable for IBP 42 litre containers. Where as 300 capacity goblets are suitable for J-12 (IBP) or LR 40 conisters.

Despatch of semen : Different types of storage containers as well as despatch containers are available in the market. The firms dealing with these containers are Union Carbide (USA), Minnesota valley Engineering (USA) L'Air Liquide (France), British Oxygen Limited (U.K.) Indo Burma petrolium (IBP) India, Daaps cryogenics, Delhi India. The storage containers best suited for bigger organisations are LR 750 LNR 500, 320 and LR 180 and freezer cum storage container LNR 250 or LNR 320. Despatch containers LD 30, LNR 25, 35, XR 16, SX 18, BA 20 etc., and for field use AL 3, BA 3, CA 3, 5, 10, or Miniwonder 1 are available. At the BAIF the pattern of distribution of container is as follows :

At sub-centre one 50 litre liquid nitrogen storage container is supplied along with AL 3, a 3 litre container for holding small number of straws for door service to cows on call by farmers. The inseminator is supposed to return back to his centre. The semen can be held in this container for 10 to 15 days with sufficient liquid nitrogen but every 3rd day the liquid nitrogen needs replenishment. Along with these two containers XR 16 (29 litre capacity) liquid nitrogen refrigerator is supplied for bulk storage of semen. This accomodates 1920 straws and has six canisters each carrying 4

goblets of 80 straws each. AL 3 container carries 6 conisters of 2 goblets of 80 straws each. Frozen semen straws from one container to another container should be transferred along with the goblet as quick as possible. Possibly with in ten seconds.

Thawing of semen : Cassou (1970) recommended thawing of frozen semen in straws at + 35° C for 15 seconds in water kept in a thermos flask. It is also advocated to use disinfectant to the thawing water especially in period of epidemic disease. The straws should be quickly fitted to the insemination gun previously warmed by rubbing vigorously to avoid cold shock. He recommends against thawing of two straws at a time and preparing more than two syringes at a time. Any fall in temperature after thawing of semen should be avoided. While thawing semen following steps should be considered.

* Straw should be removed with the forceps from the flask.
* The straw should be shaken vigorously once or twice to remove liquid nitrogen from the cotton plug.
* The straw should be placed in warm water at 35°C in thermos flask for 30 seconds. The straw should dip completely in water.
* Dry the straw with a tissue paper also wipe the scissor.
* Warm the chamber of the insemination gun by rubbing vigorously.
* Hold the straw vertically with the cotton plug downwards.
* Cut the straw at right angle to remove the powder plug or sealed plug through the air space.
* Withdraw the piston of the syringe.

* Place the straw in the warmed up chamber of syringe.
* Take the sheath from the container and fix the sheath over the straw to ensure the firm union between the straw and sheath.

Aamdal and Anderson (1968) reported that if the straws were thawed at 75° C for 12 seconds, the recovery rates were higher than thawing at 35°C Almquist and wiggins (1973) used two thawing temperatures of 5° C and 75° C & found, if straws were thawed at 75° C for 12 seconds the recovery rates were higher by 10 to 12 per cent. Rodriguez et al (1975) reported that the post thaw motility improved as the thawing temperature was increased from 5° to 55° C ($P < 0.1$). Further increases in thawing temperature to 90°C did not enhance the survival, but for all practical purposes the thawing temperature of 35° C for 15 to 30 seconds is recommended, since if inseminator by mistake is not particular about thawing time the semen will get unnecessarily warmed up and the motility will be reduced.

According to Amann and Pickett (1984) thawing of frozen semen at 35° C water for 30 seconds gives 4 to 10% higher fertility than the frozen semen thawed at 35° C for 12 seconds as usually recommended.

For thawing semen, water bath at 35° C for 15 sec. 50°C for 12 sec. 65° C for 11 sec. and 75° C for 9 sec. and 95° C for 7 sec. was used by Wiggin and Almquist (1975). regression analysis revealed that for each 20°C rise in thawing temperature over the range intact acrosomes increased a mean of 6.6 and 4.4% units. While a sperm motility increased a mean of 2.4 and 6.6 percent units at 0.5 and 2 hr. equilibration

respectively. Similar were the results by Platov et al (1977) and Ruggs et al (1977). Ahmed (1974) used water bath temperatures as 0° C, 37° C and 75° C for a min., 15 sec. and 9 seconds respectively. The post thaw motility obtained was significantly higher at 75°C for 9 seconds.

Hube and Uribe (1980), Vera (1980) Neumann Warner (1980) who used 40°, 17° C and 5° C as thawing temperatures for frozen semen, showed that semen thawed at 40° C had significantly higher forward motility of spermatozoa with high percentage of normal acrosomes than semen thawed at 17° C or 5° C however the other groups i.e. 17°C or 5°C did not differ from each other. Pace et al (1981) evaluated frozen semen thawed at 1° to 3° C (iced water) 5° to 20° C (ambient) and 37° C. According to them semen thawed at 37° C was significantly better than other two temperatures. All the semen quality test like progressive motility, intact acrosomes and capacity of spermatozoa to pass through sephadex filter was significantly better in semen thawed at 37° C than other temperature. ($P < 0.05$) Bhosrekar et al (1984) observed higher post thaw motility, live count and intact acrosome when frozen semen was thawed at 37° C as compared to frozen semen thawed at 5° C or 25° C. On incubation at 37°C for 2 hours the live count and motility was higher till 60 minutes in semen thawed at 37° C while it declined faster at other two temperatures after initial rise on 5 min. incubation. Bhosrekar et al (1988) observed 50° C for 15 seconds as optimum thawing temperature and time for buffalo semen, however they advocated 40° C temperature for 30 seconds for all practical purposes in the field.

Facilities required For establishment of Frozen semen laboratory and utilization of frozen semen.

A.I. Equipment at Sub-Centre :

a) Liquid nitrogen storage tank	Lab. 50 / TA 55
b) Frozen semen storage tank	XR 16 OR SX 18 / CB20 / Super 20 IBP
c) Transport container for frozen semen for A.I. work.	AL_3 BA_3 / CB_3 or Miniwonder 1 lit. (IBP)

d) Insemination guns : 2 universal guns/Minitube or French gun for Mini straw or other straws as per the policy.

e) A.I. Sheaths with universal sheath container sheaths for Minitub or French medium straws.	As per requirement

f) Motor cycle suitable Yezdi or Rajdoot 1.75 or Bullet 3.5 HP for village roads.
g) Container carrier to be fitted to motor cycle. Thermos flask. Roaps, Soap and Towel.

The above equipment is required to run one cattle breeding centre. In addition to this following office equipment is required.
Table - 1, Chairs 2, Stool 1, Bench 1, Cupboard 1.

Equipment for Semen Lab.

Most essential equipment

1. Hot air oven	1
2. Autoclave	1
3. A.V. sterilizers	2 (24 Avs)
4. Triple glass distillation unit	1
5. Photo electric colorimeter EELor spectrophotometer with auto pipette with 3 to 4 dozen cuvettes,	
6. Water bath/incubator	1
* 7. Phase contrast microscope with warm stage (Nikon)	1
8. Refrigerator	2
* 9. Cold handling cabinet	2

*10.Filling and sealing machine for German straws.	
*11. Straw printing machine	1
(Can be used for both French midi and German minitubes)	
12. pH meter (digital) single electrode	1
*13. Single pan electronic balance (Metler)	1
14. Freezing ramps for 100 strawsFrench or 140 German minitubes	100
15. Straw distributor for French straws.	1
16. Freezing grill	2
*17. LNR 250 OR 320 for semen freezing and storage	2
*18. Straw collecting forceps	2
19. Goblet stands	2
20. Goblets as per requirement for 300 straws/80 straws etc.	
21. Woolen gloves	2 pairs
22. Leather gloves	2 pairs
23. Printing ink, black	As per requirement
*24. Double sided sticking plaster	2 rolls
25. Thinner	2 tins of 500 ml.
26. Acetone for cleaning ink	
27. Rubber printing blocks as per requirement	
28. Glassware Collection tube	3 dozen or more
Flat bottom flask	150 ml. 3 dozen
Cylinders graduated	50 ml.
	100 ml.
	200 ml.
Petridishes	2 Doz.
Syringes 5 ml.	2 Doz.
Beakers	2 Doz.
29. Rubber wares : Avs 10"	24 Nos.
Avs 12"	24 Nos.
Av linings smooth (rubber)	50
" rough (rubber)	50
Av cones (rubber)	100
Rubber straps	100
Hand gloves 7 No.	50 Pairs

30. Chemicals :

Tris	As per requirement
Citric acid	- do -
Fructose	- do -
Glycerol	- do -
Sodium chloride	- do -
Sodium citrate	- do -
Sulphuric acid	- do -
Potassium dichromate	- do -
Dettol	- do -
Methenol	- do -
Savlon	- do -
Alcohol	- do -
Labolene for moping floor	
Pottass ium permangnate and formaldehyde for fumigation.	

Miscellaneous equipment :

Bull aprons
Gum boots
Aprons for Lab workers
Masks and head cover
Slippers
Rubber matting
Coir matting
Collection crate
Nose rings
Eggs fresh etc

Items marked * can be obtained from

1) M/s Minitub gmbh Abfull und Labortechnik Haupstraße 41, 8311Tiefenbach b. Landshut West Germany

2) IMV, 10 Rue Clemenceau B. P. 76 , 61300 L' Aigle, France

3) M/s Minnesota valley Engineering 407, 7th street N.W. New Frague Minnesota 56071 USA

4) M/s State bourne Cryogenics, U.K.

Equipment needed to bring more objectivity in processing of semen

* Photometer with auto dilutor and Printer (Microcell counter)
* Computer Assisted Semen analysis system. (CASA)
* Computerised semen freezing system "Digitcool"/ "Planner"
* Laminar flow to keep away contamination in semen processing, ultraviolet lamps on working tables and in hatches
* Interference constrast attachment to microscope.

If French technique for semen processing is used :

1. Ultra sonic semen filling and sealing machine MRS 3 x 3 or 1 x 1 for French medium straws.

Or

MRS 3 x 3 or 1 x 1 for French mini straw and spares for the above machines.

2. Printing machine for French straws.
3. Vacuum pump, straw clips, filling combs, bubbler dish assembly, PVA powder for sealing straws in absence of automatic machine.

Setting up a Semen Freezing Laboratory :

It is optional to have a liquid nitrogen plant, but as a standby one plant of either 10 lit. or 25 lit. capacity should be purchased and installed at centralised place (head quarter). If it is phillips or Sulzer plant, 10 lit. plant comes with built in chilling unit. Earlier the plant is to require a separate water chilling assembly for circulating chilled water through plant. For optimum functioning the chilled water at 7 to 10° C should be

fed. Liquid nitrogen plant needs thorough maintenance.

Now a days in India the liquid nitrogen is available as a by-product from fertiliser factory at cheap rate though the liquid nitrogen may not be cent percent pure yet it can be equally efficient to preserve the frozen semen.

If it is decided, to have a liquid nitrogen plant, it requires a room of 20' x 20' x 15'. Foundations, to put cryogenerator and electrical panel board to have the switches and guazes to control the plant. It also requires "Hydrogen gas" which works as a nitrogen liquifier with cryogenerator.

Along with the building for liquid nitrogen plant, a hall of 25' x 25' x 15' should be provided to store the containers of liquid nitrogen. Along with it another room of 25' x 25' x 15' should be provided as a semen bank to hold frozen semen. (Bulk Semen Storage Tanks). The semen bank should be very close to semen processing and freezing laboratory but it should not directly open into it. A corridor should seperate these two buildings.

The semen laboratory building should be divided into five rooms. The enterance to laboratory should never be direct to avoid contamination. The passage leading to laboratory should be provided with airlocks to reduce down contamination.

From passage, doors should open inside to different rooms. On one side it should have 3 rooms. The first room near enterance should be glass washing room with sterilization equipment like autoclave, hot air

ovens etc. it should be provided with exhaust fan, 2nd room after a first air lock should be AV preparation room. It should be provided with hatch having double glass door to deliver prepared AV. It should accommodate AV sterilizers, geizer for hot water, tube warmer etc. It should also have a exhaut fan, the third room in a front row should be semen processing and freezing lab, after a 2nd air lock. This room should be spacious and able to accommodate instruments like cold handling cabinets, refrigerator, filling and sealing machines, printing machine, computerized biological freezer, microcell counter, microscope, laminer flow table. It should be provided with sunmica top tables. The working tables should never be of brick and mortar or of glazed tiles. This room should have a double glass door hatch for receiving semen. The 3rd air lock will lead into a blind room for change over of aprons, masks and caps. The worker has to change over his dress in side room provided at the entry, then enter the passage, through 3 air locks. At the end wear apron, mask and caps, come back through the passage and enter the lab. It has been experienced that by this arrangement we could keep away the bacterial load to the maximum extent. Two ultra violet tubes are provided in the ward robe where aprons are hanged.

Opposite to the lab. the passage should open into another laboratory of similar size where sperm morphological studies are carried out. The staining equipment, microscope are placed along with the tables for laboratory staff. Adjoining to the morphology Lab. extending and opening out side at the entry of Lab. is the room for incharge officer.

Both the laboratories have 25' x 20' x 15' dimensions. The passage is having 8' width. First two air locks are therefore 8' x 15' while the third air lock is 8' x 20' and 4th blind room is 8' x 10'. All the laboratories should be provided with false sealing and air conditioning. 2 airconditioners of 1.5 tons are sufficient for each laboratory. PVC flooring should be provided for ease in cleaning and minimising infection. There should not be any wash basin in the laboratory and horizontal surfaces should be minimum.

The AV room and washing room require no air conditioning they have exhaust fans to throw the hot air out side.

Laboratory Sketch See Annexure-1

To ensure proper working of the equipment following points need consideration.

Electrical Power : Most of the equipment/instruments require power supply for operation. It is essential to ensure regular supply of current for smooth operations. Regular maintenance of basic electrical equipment like switch gear, transformer, distribution boards and fuse boxes is a must. It is observed that failure of electricity supply is not a uncommon factor in our towns and hence a installation of generator to ensure continuous electricity supply should be considered.

Surrounding : Dust and dirt is the most common cause of infection and problems in instruments. The laboratory should be made dust proof. The land scapping of the surroundings of laboratories, plantation of trees is advocated. It also gives an aesthetic appearance to the building.

Entry in the Laboratory : It should be restricted to workers only. Visitors should be discouraged and strict regulations should be implemented as per drug manufacturing unit. Whenever, visitor are to be taken in the laboratory the foot wear should be changed, lab. coat/apron should be worn, cap and mask should be used. As far as possible visitors should be taken through the corridors and should be explained by showing them through glass panels.

Location : The location of the laboratory should be such that it should be away from commonly approachable place. It should be nearer to bull station and collection yard but should be separated from despatch section, where public gathering is expected. For the sake of common visitor functioning inside the laboratory should be explained with the help of photographs arranged in the panel.

The collection crate should be covered. Rubber matting should be provided for crate. The water tap should be available in collection shed for washing and cleaning after collection. The washing room should be fitted with sinks, water taps and sufficient furniture and electrical fittings.

Recommended procedure for processing of semen for deep freezing :

Selection of ejaculates for processing and freezing

While introducing the bull to regular collection schedule. Certain criteria are exercised. At collection the body weight and age of the bull is observed to be as follows :

Breed	Age	Body weight
Holstein Friesian	15 months	350 Kg.
Jersey	15 "	275 Kg.
Cross bred	24-26 "	300 Kg.
Murrah	30 "	400 Kg.
Surti	24 "	300 Kg.
Gir	36 "	350 Kg.
Kankrej	36 "	350 Kg.

For exotic bulls the semen is screened from 15 to 18 months for ejaculate volume, sperm concentration, sperm motility and sperm morphology. Similarly for cross-bred and indigenous bulls the semen screening is done for 3 months.

For introducing bull to regular collection schedule the bull should fulfill following criteria.

The ejaculate volume should be more than 1 ml. for all the categories. The sperm concentration should not be less than 600 x 10^6 per ml., the forward motility of spermatozoa should be more than 70% and abnormal count should be less than 15%. In exotic bulls as well as buffalo bulls the rejection of bulls on account of sperm motility or sperm concentration is negligible. Out of 40 Murrah buffalo bulls screened only one bull was culled on account of low sperm concentration while there was none in exotic HF and Jersey. In cross-bred bulls, however, the rejection was higher in HF cross-bred (20.7%) as compared to Jersey cross (9.0%). The cross-bred bulls are mainly rejected on account of low sperm concentration (watery semen) and high abnormal count (Bhosrekar, 1988, Mathew, 1988).

* Evaluation and Processing : As soon as the ejaculate is received in the laboratory following steps should be followed :

The ejaculate volume should be noted and the semen should be identified properly by labelling.A drop is taken on warm slide for mass activity.

0.2 ml of semen is added to 7.8 ml of sodium citrate solution for estimation of sperm concentration using blue filter No. 303 in EEL Photoelectric colorimeter, alternatively sperm concentration is estimated by autodilutor and digital photometer (IMV).

Initial dilution is done by adding dilutor to the semen tube, in the same quantity as the semen, and left for cooling to room temperature (22° C).

Mass activity is noted by putting a drop of semen uncovered by coverslip on a warm glass slide and viewed under phasecontrast microscope for eddies and waves.

Initial motility is noted under phase contrast microscope with 20/20 phase by taking a drop of diluted semen on a warm slide and covered by a cover slip. The stage of microscope should be also warm at 37° C. Sperm motility is recorded in percentage as progressive motility.

The doses of semen are calculated from the chart being followed from the initial motility and concentration. 30 millions of motile sperm per dose of 0.28 ml (minitube) is recommended. The straws should be got printed for batch No. Bull No., and Institution Code or Name.

The diluted semen is now kept out of water bath and allowed to cool down to room temperature (22° C). The semen is now diluted to the final volume before filling and sealing is done (Landshut Method). In case of French method after first dilution the semen is cooled to 5° C slowly over 2 to 2 .5 hours before glycerolisation.

The semen is then filled and sealed in landshut automatic filling and sealing machine at room temperature. The sealing is done by metal and glass balls. On one side metal ball is used and on other side coloured glass ball is used. In French method when the semen gets cooled to 5° C, the 2nd part of diluent containing glycerol is added in 3 to 5 different equal fractions with an interval of 10 to 15 minutes, and glycerolisation is completed in 45 to 50 minutes. All process is carried out at 5° C temperature and care is taken to avoid temperature rise during processing. The semen is then filled and sealed at 5° C temperature.

The straws are then arranged on freezing ramps or racks with the help of straw spreader.

The racks are then kept at 5° C for 5 to 6 hours equilibration.

After equilibration period the racks are kept over freezing grill, touching the liquid nitrogen. The height of the semen straws on the freezing racks is 4 cm. from the grill. The temperature at that height is -180°C.

Before the freezing racks are arranged on freezing grill for freezing straws, the level of liquid nitrogen is

checked in the container. It should be touching the grill. The container is covered by round hard board cover or transparent thick nylon board for ten minutes before the freezing racks are kept. After 10 minutes the cover is slowly taken out without disturbing LN vapour and freezing racks are kept and then again the container is covered.

The racks are kept on grill for ten minutes. In ten minutes the straws get frozen. The frozen straws are then collected by special forceps and transferred in already cooled goblet. Care is taken to cool all the equipment to freezing temperature before straws are handled. The goblet is then immersed in liquid nitrogen and stored in canister which is identified.

The grill should be 30 cm. below the brim. The height of canister should be 26 cm only. Six hundred straws (minitubes) could be easily frozen at a time in one freezer (LR 250) while 300 French straws could be frozen at a time.

The frozen straws should be kept in the freezer only till one month for the sake of quarantine and then transferred to other containers.

Check

Every equipment is kept ready for next processing on following day while leaving the laboratory at the evening.

Before filling and sealing machine is operated the syringes should be kept ready as many as the number of semen samples. The proper balls are fed to the respective chambers of filling and sealing machine.

The volume is properly adjusted. The syringe should be rinsed with diluent before using in semen.

Half an hour before the collection is started the water bath, slide warmer and biotherm should be put on. The colorimeter tubes should be filled with 7.8 ml of sodium citrate (2.9 per cent) solution.

Labels should be cut and kept ready.

Daily semen record card should be kept ready with batch number and date written on it.

The geizer should be put on and AVS should be kept ready.

After the processing; Check

The microscope slides, pipettes, the cylinders and semen containers, semen collection tubes are properly washed, cleaned and sterilized. The colorimeter tubes should be cleaned by acid solution or detergent solution and washed in distilled water and kept inverted on stand. The slides should be immersed in ether-alcohol mixture for removing grease after washing with water.

The syringes should be immediately rinsed with water washed and rinsed with distilled water and sterilized in syringe sterilizer.

AVs should be washed with soap water, rinsed with tap water and distilled water and then sterilized in AV sterilizer. The cones should be treated similarly.

The ink from the roller of printing machine should be washed with cleaning solution (acetone) and oil should be applied.

The filling and sealing machines should be cleaned. The straw feeding wheel, the piston pushing the balls etc., should be taken out and cleaned by alcohol. The oiling points should be regularly oiled.

Records The following types of records are recommended for daily and for storing record, bull wise.

1. Semen collection data sheet See Annexure
2. Bullwise store card See Annexure
3. Service behaviour card. See Annexure
4. Bull health card.

After freezing, check :

* Recovery rates are checked after thawing on 24 hours storage under liquid nitrogen.

* Straw is thawed one after another at 35° C in water bath for thirty seconds. Only one straw should be thawed at a time.

* One end is cut and the straw is put inverted in small 1 ml tube kept at 35° C water bath on test tube rack. The other end is then cut, so that whole semen comes in tube. The semen is then mixed and a representative small drop is taken on slide and covered by cover slip.

* The post thaw motility is checked under phase contrast microscope 20/20 phase on a warm stage.

* The samples showing less than 50 per cent progressive motility should strictly be discarded. This should be observed to maintain uniformity in results.

CHAPTER IX

CLEAN SEMEN PRODUCTION

Disease testing of bulls :

Bulls maintained at semen banks should be free from infections and communicable diseases like tuberculosis, Johnes disease, brucellosis, trichomoniasis, campylobacteriosis, leptospirosis, listeriosis, IBR, epivag and I.P.V. The bulls should be examined regularly for tuberculosis and Johnes disease every six monthly by double intradermal tuberculin and Johnin test. The positive reactors should be culled and immediately removed from the bull station. The genital diseases like brucellosis, campylo bacteriosis, trichomoniasis, leptospirosis and listeriosis should be regularly checked in breeding bulls by laboratory testing; culturing for organisms of the said diseases. The bulls should be immediately culled if found positive for either of above diseases.

For brucellosis regular checking by serum agglutination test or seminal plasma agglutination test should be carried out. For campylobacteriosis (vibriosis) preputial washings should be collected, centrifuged and the sediment should be inseminated in virgin heifers and virgin heifers should be tested for campylobactoriosis after development of disease (six weeks) by taking cervical mucus and carrying out mucus agglutination test with campylobacter antigen, or the preputial washings cultured in laboratory, using special media and campilobacter being identified under microscope. Trichomoniasis also can be checked by collecting preputial washing and

examining for the presence of flagellated protozoa under microscope. Leptospirosis as well as listeriosis could be diagnosed by biological tests using laboratory animals.

Bulls should also be regularly examined for the viral epididymitis and other virus organisms causing pustular vaginitis in female. IBR-IPV infections can be diagnosed by Elisa test.

The semen should be free from all those organisms. Strict hygienic measures should be taken at the bull stations. The prepuce and sheath should be regularly washed by mild disinfectant lotions for minimising bacterial count in semen.

Injury on any part of the reproductive organ of bull should be avoided by providing soft bedding to the bull. The injury may serve as a source of infection. Seminal vesiculitis, epididymitis or orchitis may be sequel to ascending or discending infections.

Tumors or absceesses on urethra or glans penis of bulls may lead to serious consequences and such bulls should be immediately culled. The bull pens should be thoroughly cleaned every day with disinfectants and should be sprayed with tickicides.

The humidity should be avoided in bull pens since organisms of infectious diseases hang around in humid atmosphere. The bull pens should be well ventilated and clean water should be made available. In winter the bull pens should be provided with soft bedding to avoid frostbite to the scrotum.

Since all febrile diseases reduce the sperm production and cause increased abnormality in spermatozoa, immediate attention should be given to bulls suffering from systemic diseases.

The foot and mouth and other viruses appear in semen during incubation period of the disease before the bull actually suffers from clinical symptoms and since the foot and mouth as well as other virus like IBR, IPV can survive under liquid nitrogen and can cause disease on use of frozen semen, the frozen semen should be kept under quarantine for one month before despatch to field centre.

International standard for production of semen : The bulls should be free from brucellosis, campylobacteriosis trichomoniasis, leptospirosis, listeriosis, epivag, IBR and IPV. Tuberculosis and Johne's diseases should also be tested regularly and positive reactors should be culled. The semen should have minimum bacterial count. The certificate has to be given to this effect if semen has to be exported. The semen should not contain pathogenic organisms.

Sterilization of equipment : The Artificial vaginas should be meticulously sterilized in A.V. sterilizer and dried and packed in polythene or by aluminium foil cover and should be opened only just before collection. Similarly the cone and glass semen collection tubes should also be sterilized properly. All glassware coming in contact with semen during evaluation processing and filling should be completely sterilized.

Use of bull aprons and towels : Bulls should be used with clean bull aprons during collection of semen since the penis should not touch the hind quarters of dummy animal and get contaminated. Each bull should

be provided with his own apron. The aprons should be cleaned regularly. Each bull should also have a separate A.V. and each ejaculate should be collected in separate A.V. Each bull should have seperate towel for its cleaning before collection.

Preparation of diluents : The eggs used for getting yolk should be from birds (hens) free of disease. The eggs should be fresh. The eggs should be cleaned by alcohol and tapped to drain out white of egg. The egg yolk should be taken on sterile filter paper and should be punctured by sterile forceps and then collected in sterilized cylinder. The whole process should be carried out in sterile zone caused by naked flames from two gas burners. The distilled water used for preparation of diluent should be triple glass distilled water and should be sterile (autoclaved). Antibiotics at the rate of 1000 I.U. of penicillin G. Sodium per ml and 1.5 mg per ml of streptomycin sulphate should be added to the final diluent to check the post contamination in diluent.

Cleaning of filling and sealing machine : The filling and sealing machine used for packaging the semen should be thoroughly cleaned before use with alcohol.

Sterilization of straws : The straws used for packaging semen come as sterilised from factory but handling of straws for printing etc., can contaminate their outer surfaces. Therefore, the straws should be sterilized by ultra violet light before use for semen packaging.

Site of collection : The site of collection of semen should not be dusty and should be away from public place. The collection shed should be permanent

(pucca) and rubber matting and coirmating should be provided on the floor for jumping of bull. After collection is over the shed should be washed and cleaned and kept tidy.

Laboratory : The laboratory should be dust proof, clean and air conditioned. The temperature of laboratory should be 20° C to 22° C. Visitors to the laboratory should be avoided and workers should change over the shoes and wear aprons before they enter the laboratory. The laboratory enterance should not be direct but through 2 to 3 airlock rooms. Fumigation with formaldehyde-$KMNO_4$ should be carried out every week especially on saturday. So that the whole laboratory and, the passages get exposed to fumes.

Bacterial load in frozen semen : Contamination of fresh and preserved semen with microorganisms poses a great threat to successful AI programme. Aseptic collection of semen under our conditions seemed to be highly impracticable. Even under careful conditions the semen might get contaminated at the time of collection or subsequent handling.

Place and Kasde (1961) reported reduced fertility of bull semen contaminated with corynebacterium Pyogenous, where as Sakale et al (1961) reported spermicidal effect of E.coli. Roberts (1971) observed deleterious effect of bacterial load on fertility of semen.

Various measures should be taken to minimise bacterial load. The bulls should be thoroughly groomed and washed with water on the previous evening and in the morning before collection. The loose hair should be removed. The sheath should be washed with

warm distilled water and cleaned with clean towel. Every bull should have its own towel to avoid cross contamination. Before collection and giving false mounts, the bull should be tied a bull apron to avoid penis getting soiled by rubbing against hindquarters of dummy animal.

The AV used should be properly sterilised after cleaning and washing etc. Each bull should have its separate bull apron and AV. The AV should not be used more than once. Even if second ejaculate is needed to be collected separate AV should be used.

The glassware coming in contact with semen should be thoroughly washed with detergent, distilled water and sterilised in hot air over at 110°C for half an hour.

The media like tris buffer should be autoclaved before its use for preparing diluent. The egg yolk should be prepared from fresh hens' eggs. The eggs should never be washed with water and should be cleaned with alcohol before breaking. Older eggs beyond 3 to 4 days should be rejected. Therefore, egg purchase should be done twice weekly and fresh eggs should be purchased.

Extracting egg yolk should be done in presence of naked flames in sterile zone. The forceps and hands should be cleaned with alcohol. The shell should be broken in two halves, the white of the egg should be drained off in a beaker. The yolk should be taken on a filter paper. The intact yolk is dried free from white by rolling over a clean filter paper. It is then punctured by forceps to drain yolk in a sterile cylinder. Required quantity of yolk is prepared and kept covered with aluminium foil for preparation of diluent.

The diluent is prepared in sterile triple glass distilled water by weighing meticulously the tris, fructose, citric acid as per the composition.

The laboratory should be fumigated once in a week with formaldehyde by using $KMNO_4$ in petridishes or by 'Aerosol'. Every day the laboratory is wet mopped with disinfectant. The working tables should be cleaned by dettol lotion mopping. Ultraviolet lamps should be fitted over working tables and the laboratory should be exposed to U.V. lamps at least one hour before starting of work and after finishing of day's work. The effective life of UV tube is only 200 hours. Therefore the UV tubes should be replaced after every 200 hours of exposure.

As far as possible venyl flooring (poly vinylchloride) should be got fixed on the floors of the laboratory to avoid crevices or cracks on the floors which can serve as hiding places for bacteria and insects. The laboratory should be dust proof and air entering the laboratory should be filtered. Laminer flow tables should be used for processing and filling and sealing operations of semen.

Entry to visitors should be restricted. The people working inside the laboratory should use aprons, head caps and masks. The shoes should be left outside and on entering laboratory clean slippers should be used.

To avoid dust around laboratory, the lawns should be prepared and to break wind, trees should be planted. The collection yard should be pucca with walls and roofs.

By observing all these precautions, it will be possible to produce 85 to 87% of the frozen semen doses free from bacterial load and 100% absolutely free from pathogenic organisms. The recommended ISI standards for bacterial content of frozen semen is 1000 non pathogenic bacteria per ml. of frozen semen.

CHAPTER X

CARE AND HANDLING OF FROZEN SEMEN EQUIPMENT AND FIELD APPLICATION OF FROZEN SEMEN

Care and maintenance of containers :

* Avoid dropping or jolting the vivostat
* Avoid forcing the plug cap or canisters
* Tilting or putting it on its side leaving the plug cap off should be avoided
* Avoid pulling or pushing the vivostat along the ground
* Handle carefully the vivostat and remove or replace the canister easily
* Always keep the vivostat in vertical position and while lifting, lift with handles only
* Never keep the vivostat open for long time, dirty air, carbondioxide and oxygen will enter the vivostat and cause damage.
* The neck tube of the container is the most vulnerable part and can get damaged by mishandling and can cause leakage of the container.

Routine maintenance of vivostat : A vivostat in continuous use should be emptied of liquid nitrogen every twelve months and left to warm up over a week. The canisters and plug is removed and gaseous dry nitrogen should be flushed out for one hour. This procedure prevents to build up the contaminants (including oxygen) in vivostat. Paint the vivostat when the paint is lost, with special low temperature resistant paint.

Field application of frozen semen for artificial insemination :

Some salient points for consideration -

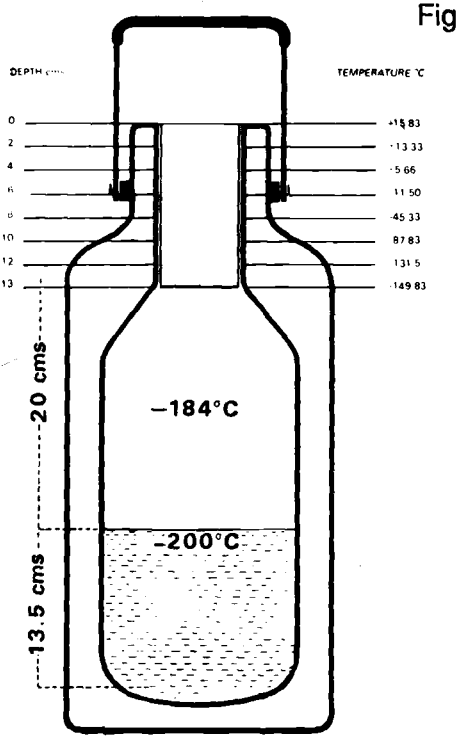
* Frozen semen should be properly handled once it is supplied to field staff.
* The semen straws should not be exposed to sun or wind.
* The container should not be kept in sun or rain.
* Straw should be quickly taken out one at a time with the help of forceps and not by fingers.
* It should be shaken once or twice vigorously so that liquid nitrogen from plug is removed
* Thaw the straw in warm water at 35° C in thermos flask for 30 seconds
* The insemination gun and other equipment should be kept dry and warm at 35° C to avoid cold shock after thawing.
* Straw should be wiped and dried with tissue paper The scissors also should be dry.
* The straw should be held with the cotton plug down wards.
* The straw is then placed in chamber of gun previously warmed up by rubbing.
* The sealed end is then cut through air bubble and the sheath is then placed over the straw and fixed neatly.
* The semen should be utilized within five minutes after thawing. The container should be taken as near to the animal which is to be inseminated as possible and all the above procedures should be adopted.
* While taking out the straw the canister should not be lifted above the frost line of the neck of the container for longer time and the canister should be immediately lowered in the container under liquid nitrogen.

* The inseminator should have a clear understanding of the basic principles of handling semen, liquid nitrogen and LN refrigerator. Training and retraining of the inseminators are essential for any AI organisation.
* Prime importance should be given to replenishment of liquid nitrogen in LN containers as per schedule.
* A uniform procedure for thawing should be adopted, preferably a waterbath at a temperature of 35°C for 30 seconds should be used. Palm thawing or pocket thawing should be discouraged for getting optimum results.
* The frozen semen once thawed should be utilized immediately within 5 minutes. Thawing frozen semen and holding in warm water for more than half an hour should be avoided as aging process of spermatozoa starts resulting in poor fertility.
* The frozen semen should always be kept under liquid nitrogen. The critical level of liquid nitrogen and the temperature pattern in the container will be as follows :
* In AL3 or BA3 container, the critical level of liquid nitrogen is 10 cm. That means if the liquid nitrogen level goes below 10 cm. the lquid nitrogen should be immediately replenished. (Fig. 23)
* The frozen semen should never be taken out of container unless the cow is examined per rectum and confirmed in heat.
* The frozen semen after thawing should be deposited half a centimeter ahead of internal opening of cervix.

If the canister is lifted out of vivostat containing liquid nitrogen it immediately starts warming up. This causes following disadvantages :

* The canister contents will warm up and may become damaged
* When a warm canister is returned to the vivostat a heavy loss of liquid nitrogen will result
* The ice formed on the canister will drop off into the vivostat, when canister is replaced causing an undesirable sludge to build up.

Fig. 23



If the vivostat is kept open for long time ice formation takes place on the neck of the tube and when the cap is being replaced it falls in container causing damage.

The straw should be taken out by forceps. Do not press too much the forceps otherwise the straw will

break as the straws become brittle while in liquid nitrogen. Care shoud be taken not to drop the straws in container as it would be very difficult to take out the straws and an attempt to take out the fallen straws may damage the container.

The container cork should be put in position immediately after taking out the straw. If delayed the cork becomes wet because of the water of condensation and if put in without wiping gets fixed because of ice formation. Therefore, cork should be placed in position only after thorough drying by towel.

Frozen Semen is one of the most advanced and sophisticated technique in livestock breeding and its impact on rapid improvement of cattle is beyond doubt.

Advantages of artificial insemination

Artificial Insemination is accepted as a tool for quick genetic improvement in cattle and other species of livestock because of several advantages, which score over the limitations.

1. It helps in dessimination of best available germ plasm to more population of livestock at a time to get improvement quickly, effectively and economically.
2. It helps, small marginal farmers and landless labours to avoid keep bulls for themselves thus cutting down expenditure on maintenance of bulls.
3. It helps in reducing the incidence of venerial diseases, which spreads most often through natural service.
4. It helps in providing more complete and accurate breeding records, which has led to better herd management.

5. It helps in detecting organic changes in reproductive tract of female leading to infertility, so that timely treatment is possible.
6. It helps in breeding disabled cattle having good records. e.g. good high pedigreed cows physically handicapped for bearing the weight of bull can successfully be bred and she can deliver good calves. Similarly good top sire but disabled can be utilised by collecting his semen by means of A.V. or electroejaculation etc., which otherwise could not be used for natural breeding.
7. Semen can be flown from one country to other with success, and over longer distances within the country.
8. Through advant of deep freezing technique the sires even could be disposed off or slaughtered. Thus reducing the maintenance cost and at the same time the semen is available for utilization.
9. Artificial insemination helps in early progeny testing of a sire.
10. A check on semen quality can be exercised through this method.

Advantages and limitations of frozen semen

1. Deep freezing of semen has been introduced as early as 1949 by Polge, Smith and Parkes. The advantages are many :
2. The semen can be preserved indefinitely under liquid nitrogen without loosing fertilizing capacity.
3. Semen can be preserved from number of bulls simultaneously in one container.
4. Required number of doses can be collected from the bull and bull can be disposed off or slaughtered. The progeny can be born even if the bull is no more living making the programme more economic.

5. Number of bulls can be used for Artificial breeding simultaneously. which is not possible in case of liquid chilled semen and hence progeny testing of bulls will be much more easier with frozen semen technology and better utilisation of outstanding bulls.
6. Frozen semen can be transported with less cost from one continent to other and one place to other place with in the country with great ease. Frozen semen can reach remotest place in the country without losing fertility which is not possible with liquid chilled semen. More rational and convenient planning of daily work in semen banks can be carried out. In frozen semen the spermatozoa go in suspended animation therefore the motility of frozen semen remains as good as fresh semen provided liquid nitrogen is replenished and frozen semen is kept under liquid nitrogen level. Planned breeding programmes and contract mating is possible only with frozen semen.

Limitations :

It is a costly technique. To establish frozen semen laboratory at least Rs. 10,000,000 are required. This includes cost of building, equipment, liquid nitrogen plant, cryogenics etc.

It requires training of manpower thoroughly in order to reach perfection in handling of frozen semen, A.I. with frozen semen, thawing technique and deposition of semen etc.
1. Success of artificial insemination depends upon accurate detection of heat. A.I. is therefore, of limited value where people cannot have close observation of

their cows. It is really not applicable in areas with poor means of communication.

2. The risks of inbreeding are involved, which will cause spreading of undesirable characteristics or recessive genes, which may prove harmful in future breeding programme (spreading of hypoplastic overies amongst females and hypoplasia of testis in male is one of the examples of inbreeding dangers). To avoid this 33 % of the bulls should be changed each year.

3. If bulls are not regularly checked for genital diseases, there is danger of spreading disease through A.I.

4. If A.I. equipment is not properly sterilized, it may cause more harm to cattle industry and artificial infection will be introduced rather than artificial insemination.

5. Skilled personnel is one of the greatest limitations.

6. Costly equipment is another limitation which comes in way of popularising A.I.

7. Availability of liquid nitrogen, transport facilities and equipment for storage of deep frozen semen should also be looked in as limitations in use of A.I. technique by deep frozen semen.

8. Frozen semen can be potent source of dessemination of genetic defects if selection of bulls is not properly and meticulously done. Frozen semen also can pose a potent danger for transmitting diseases like IBR, IPV, Campylo bacteriosis as well as Brucellosis if thorough screening of bulls and regular testing is not undertaken. Viruses like Foot and Mouth as well as Rinderpest can pass through semen therefore regular vaccinations against Foot and Mouth, Rinderpest, Haemorrhagic septicaemia, Black quarter etc. should be carried out, and frozen semen should be held in Quarantine for 30 days before it is released for use in A.I.

CHAPTER XI

MAINTENANCE OF EQUIPMENT

To ensure production of good quality product good equipment and qualified staff is required. Smooth handling of instruments determine smooth functioning and trouble free operation of the equipment. This is possible when all due precautions are followed at all the times. Cleaning of equipment/instrument, timely check-up will eliminate many troubles.

Useful tips for maintenance of some important laboratory instruments.

1. Waterbath

* Use only soft water/distilled water if the tap water is hard. Get the tap water tested for hardness.
* Check the water level every day.
* Change the water if dirty.
* Scrub and clean all the surfaces that are in contact with water once in 15 days using Soda and a plastic brush.
* Check the temperature every day and set the thermostat for adjustment.
* Place the waterbath in a dust free area.

2. Microscope

* Keep covered with a polythene cover/acrylic hood when not in use.

  Never use cotton to clean surfaces of eyepiece/ objective or any glass parts.

  Never use harsh chemicals/acids for cleaning.

  Use Whatman lens cleaning tissue or cleaning paper for cleaning all glass parts.

* Be careful to avoid contact of objectives with semen. If there is driedup semen, use hot distilled water to clean.
* For cleaning use soft brush of '0' or '1' number. Never use any hard objects.
* To remove greasiness, use mixture of methanol 3 parts and absolute alcohol 1 part.
* Never touch the bulb of microscope with bare hands. Use plastic/polythene films to hold in bulb while changing.
* Follow the steps of instruction manual to get best results.
* Check phase adjustments periodically.
* Use voltage stabilizer of good quality.
* Check and set temperature of biotherm.

3. Colorimeter/Spectrophotometer

* Should be well protected from dust.
* All the cuvettes should be sparkling clean.
* Cuvette should be washed a number of times to keep clean. Avoid brushing.
* Do not touch the surface of cuvette with finger or hand. Hold only the upper part of cuvette. Use tissue paper to hold the cuvette while cleaning.
* Do not use rack or stand to hold the cuvette instead use thermocool stand for it.

4. Auto pipette

It would be preferable to use auto pipette instead of glass pipettes to eliminate human error. In addition, the time required for estimation of concentration is reduced to a great extent. To obtain correct result the auto pipette has to be used carefully. The disposable tips are reused routinely and require regular cleaning with distilled water and autoclaving. It is essential to rinse the tip with semen before taking the sample. Each tip should be used for only one sample.

5. Filling and Sealing machine (French type)

* The filling and sealing machine requires lot more cleaning and maintenance than any other machine.
* Assemble the needles, rubber tubes etc correctly.
* Due to handling of semen, the spillage of semen canot be avoided. To ensure trouble free operation, the cleaning of machine immediately after use is of utmost importance. Use only hot water and tissue paper. Avoid cotton.
* The detailed instructions are mentioned on the machine as well as in the manual. It is essential to follow the instructions.
* Usually the rubber tubes etc are re-used in our country. In case of reuse, ensure thorough washing in water, rinsing in distilled water, drying and autoclaving. All the needles and tubes should be autoclaved every day. As many sets of long needles with tubes should be available as the number of bulls to be collected in a day.
* The machine should be sparkling clean with no traces of dried extended semen.
* Before assembly of needles and tubes etc. clean your hands with spirit and dry.
* Keep the machine under ultra violet lamp. Use the lamp for 1 hour before the machine is put to use. Put off the lamp while working with the machine.

6. Cold handling cabinet :

The conventional cabinet has all the machineries fitted in the cabinet. Now a modified version is available where all the machines like compressor, condensor coil etc are placed outside the cabinet i.e. split unit. The advantage of this type of unit is that all the heat generated to cool the cabinet is dispersed out side. In

the conventional model the heat is dispersed in the room and thus the room temperature increases. To compensate this temperature rise, we have to operate an extra A.C. In our country, it is preferable to go for the split unit. With this unit, noise is also eliminated.

The condensor coil has to be cleaned every 3 month using a blower.
As far as possible, the semen should not be allowed to spill. Periodic fumigation will be useful.

7. Autoclave / Artificial Vagina Sterilizer
The quality of water determine longivity of heating elements. Only soft water should be used. The level of water should be checked every day. Gasket needs replacement every 6-8 months (in case of Auto clave). If deposition of minerals takes place, diluted solution of Oxalic acid (10-20%) can be used. Keep the heating elements immersed in the solution for 30-45 mts and scrub with plastic brush. Later, remove solution and change water.
All electric connections should be regularly checked.

8. Biological Freezer
Use of a servo or solid state voltage stabilizer is a must for freezer. The microprocessor gets damaged due to erratic power supply. After every operation, the unit has to be warmed up and dried completely. Do not move the LN tank during operation.
Lubrication of blower motors is essential. Refer the manual for details.

9. Balance
The balance has to be checked for accuracy periodically. After every use, it is essential that all the chemicals should be removed. It would be preferable

to enter into an annual service contract with a reliable agency for maintenane and calibration. Three to four services in a year are ideal.

10. Hot air oven

The temperature has to be checked regularly. It is convenient to use dial thermometer/digital thermometer.

The oven should not be opened when it is very hot. The vent should always be kept open. If closed, all the paper etc. used for covering glass ware gets charred.

11. Refrigerator

Needs regular defrosting and cleaning. Fungus should not be allowed to grow. At least once a month, it has to be stopped and cleaned thoroughly. Avoid spillage of stains/dilutor. All the bottles need proper labelling. Clean the refrigerator every day with detergent, methonol to avoid bacterial growth.

12. Frozen Semen storage container

Due to regular exposure of the LN to air every day, lot of ice formation is observed in such containers.

They need regular cleaning at least once in a year. Usually large quantity of grey coloured scum is observed. Transfer all the frozen semen doses into a fresh container. Use only plastic items for cleaning to avoid any damage to the container. Check all the storage container for any moisture or the ice formation on surface and neck. Presence of the ice/cool surface indicates vacuum loss. If observed in time, change the stock of frozen semen doses into a fresh container. Always keep extra containers in stock.

13. Maintenance of straw filling and sealing machine (German MT 65) and Printing machine :
The filling and sealing machine has following critical points where it requires meticulous maintenance :

1. Feeding wheel.
2. Straw feeding remote control.
3. Volume adjustment and speed control.

1. The level of straw feeding wheel should have proper allignment with the nozzle of filling syringe or pump. Keep the empty straw in the groove of the feeding wheel and move it side ways. If the allignment is correct, it will match with the boring provided for syringe nozzle. If the allignment is not correct the level has to be adjusted. For this; loosen 3 screws of transport wheel, keep the transport wheel arrested by hand as much as necessary to put grooves of straw feeding wheel in central position to the borings, then fix the screws and check again the position of straw feeding wheel after fixing the screws.

2. Straw feeding control watches the continued transport of straws. If one of the groove of feeding wheel comes empty the machine stops. Each straw passing the "feeding control" will press feeding control switch lever thus making contact provided the main switch is on "Autom" Position.

Straw feeding control does not work when main switch is on "Hand" position. One can operate machine by pressing the "Start" button continuously as long as one wants machine to work without straw in feeding chamber.

If you find that machine stops suddenly during filling process check if the groove on the feeding wheel is empty. If so turn straw feeding wheel backward until empty groove is filled with straw. For this operation the ratchet lever on the left side of filling machine has to be lifted. After the empty groove is filled turn straw feeding wheel in operating direction until empty straw is in correct position infront of semen injection boring.

Even if the straw feeding wheel has straws in all grooves the machine does not work on "Autom" switch, for this, check the straw chamber front panel where straw feeding control is fixed. Do not tighten too much the screws of straw chamber front panel otherwise function of rubber slides under screws is eliminated.

Check

* If control lever is pressed by straw.
* Take the upper end of front panel and try to move it gently 2 mm.
* Listen to clicking sound of the control switch.

Feeding wheel has to be treated carefully to avoid damages, (Sharp edges may damage straws).

The straw feeding wheel and sealing ramp should be kept clean.

Volume adjustment and speed control :
Volume of semen can be regulated by turning the semen volume regulating wheel. Regulate speed of filling machine to get the semen portion right in the middle of the straw. The straw should not be completely

filled. In order to have a 3 mm air bubble left at both ends of straw. The speed of the machine should be properly regulated.

Interruption of ball transport :

* Check sufficient content of sealing ball chamber.
* If ball filling tubes are filled yet sealing balls are not introduced into straw. Loosen sealing ram fastening screw and pull out sealing ram by hand. Clean it by alcohol and see if it properly pushes the sealing balls by hand operation. Fix it again in original position.

Cleaning of straw feeding wheel :

Remove front panel of straw chamber. Turn by hand straw feeding wheel in suitable position with wheel on left side on filling machine. Pullout feeding wheel pin. Clean with alcohol all parts of straw feeding wheel and straw chamber.

Lubrication :

Lubricate bearing of transport mechanism, shaft and levers once a week.

Setting in motion :

* Pay attention to electric power alternating current 220 volts.
* Check correct position of straw chamber front panel with straw feeding control.
* Fill straw chamber with straws. Turn straw feeding wheel by hand until first straw will be dejected.
* Fill balls in to chamber for sealing balls, cover again.
* Put into the electric counter number of straws to be filled. Filling machine will not start when counter shows 0000 or 9999. Counter is counting back ward and automatically stopping the machine at 0000.
* Put switch into "Autom" position. Press button start.

* Machine can be stopped by pressing red button.
* Before start the syringe should be checked by rinsing into dilutor for proper operation. The dilutor should be removed from syringe by several times pushing the syringe piston. Put the sinker into the semen. Full the syringe completely with semen by hand. Fit syringe into pump rod attachment.

After finishing the filling process remove the pump carefully after the machine stops, to avoid moistening of machine. Take out rest of the straws from straw chamber. Turn switch from "Autom" to "Hand". Press start button and keep pressing until last straw filled with semen is dejected. If there are unfilled straws left in the chamber, take out by turning the straw feeding wheel by hand.

14. Printing machine : Operating instructions :
1. Put a little strip of ink across the ink roller. Start the machine and when the roller has an even layer near the print roller, stop the machine.

2. Fill the magazine with straws and fix the printing block to the print roller. Start the machine and the printing will start. Remember to set the counter to 'o' before you start the printing.

If the straws stick together after printing, there is too much ink on the ink roller. This can be redressed by using a suitable quantity of diluent. The exact quantity depends on the temperature and therefore can not be stated before hand.

Cleaning the machine :
The inking unit is taken out and is cleaned in acetone. A cloth dipped in acetone can be held against ink

roller and ink roller is moved, the ink gets cleaned in a minute.

The printing blocks are required to be changed every third month as they get worn out and donot print properly. The height of the printing block should be such that it will not cause any interference in movement of print roller and at the same time will not press the straw. The printing block is supposed to have very light touch and leave inpression of print on the straw. After printing is over the rubber printing block should be cleaned by thinner and should be preserved properly in box till next use.

CHAPTER XII

INFERTILITY IN MALE ANIMALS

Factors causing infertility can be divided as follows :

* In born, usually of genetic origin
* Predisposition to functional disturbances
* Somatic diseases
* Infections of the sexual organs
* Nutritional factors
* Environmental factors
* Managemental problems or combinations of genetic and acquired factors

Inborn or genetic factors :

Hypoplasia of testes : Hypoplasia is observed as unilateral or bilateral condition at the time of puberty or later in all the domestic animals. Lagerlof (1938) observed hypoplasia to be the cause of infertility in Swedish bulls to the extent of 23%. Lagerlof (1938) reported the hereditary types of hypoplasia affecting as many 25 to 30 per cent of Swedish high land cattle. Settergren (1961) showed that the hypoplasia was associated with white colour of polled cattle in Swedish High land breed. In hypoplasia of cattle the libido may be excellent and coitus may be prompt. Lowered conception rate to complete sterility may be expected in hypoplasia of testis. In sterile bulls with bilateral hypoplasia the semen is usually clear and watery with no or few spermatozoa.

In the sediment of such semen after centrifugation giant cells, medusa cells or ciliated cells from efferent tubules may be observed. The testis are often firmer.

Histological sections show one half to two third of seminiferous tubules undeveloped. Varying degree of spermatogenesis may be present from spermatogonia, spermatocytes, spermatids to abnormal or normal mature spermatozoa.

The bulls suffering from hypoplasia should not be used for breeding. Since this condition is hereditary, caused by single autosomal recessive gene.

In bovine twins of unlike sex, the female is always sterile (99 per cent) and is called as freemartinism.

Cryptorchidism if bilateral, results in sterility in bovines. Unilateral cryptorchidism usually results in normal fertility.

Torsion of testis is very commonly seen. Torsion at 45 or 90^0 may not cause infertility but torsion of 180^0 to 360^0 may cause infertility because of interference in passage of spermatozoa.

Segmental Aplasia :

Segmental aplasia of some part of testis or epididymis or vasa deferens cause infertility if unilaterial and sterility if bilateral. The segmental aplasia has been reported by Blom (1968) in Danish Freisian cattle. Aplasia of caput epididymis has been detected in one of the Danish born Friesian bull. (Bhosrekar 1975).

Spermiostasis :

Spermiostasis naturally occuring in Danish bulls has been reported by Blom (1966).

Morphological Abnormalities :

1. Hereditary sperm defects : Bake (1945) reported narrow heads with returned tails in Jersey bulls exhibiting sterility.

Blom (1966) described Dag defect in Danish Jersey breed that had low initial spermatozoan motility to the extent of 10 to 15 per cent and very poor fertility. Strong coils of tails were observed in spermatozoa to the tune of 40 per cent. Blom (1959) described another defect corkscrew sperm defect in Red Danish, Jersey, Friesian and Aberdeen Angus breeds. This he has attributed to irregular distribution of mitochondrial sheath together with high incidence of proximal protoplasmic droplets.

Hancock and Rollinson (1949) and Jones (1962) reported in Gurnsey bulls the defect called "Lack of intact sperm cells". Free heads or free tails were observed. All heads show deep indentation marking the point of separation from the tails. The mid piece was thickened.

Abnormal Acrosomes :

Knobbed spermatozoa related to defective spermatogenesis involving the Golgi apparatus has been described in bulls causing infertility. The defective acrosome rendered the sperm cell incapable of penetrating and fertilizing the ovum. This defect is apparently due to autosomal recessive sex linked defect in Fresian cattle. Saacke and his Coworkers (1966) reported such defect in two infertile Fresian and Jersey bulls. Some studies on cytogenetics have shown that acrosome defects may occur at the time

of meiosis and result in infertility. An apparently normal semen may carry this defect. Knudson (1954) reported that certain bulls with exellent appearing semen in which genital infections were excluded had low fertility due to intrachromosomal aberrations including translocations or inversions. Gledhill (1966) showed that there is no difference in mean amount of DNA between fertile and infertile bulls. The bulls, he examined had essentially normal semen picture except slightly lowered average motility. The infertility may be due to altered immature or atypical basic nuclear protein due to defect in sperm cell chromatin occuring during spermiogenesis that interfered with normal penetration of spermcell into ova and/or activation of ova. "Stickiness" of chromosomes has been described by Knudson (1954) as a cause of infertility in Swedish lowland (Fresian) cattle. The semen centrifugate had giant cells and pyknotic nuclei. Probably a recessive gene is responsible for such a defect which was predicted by Knudson (1954) since all 5 bulls of his study belonged to the same family.

Morphological abnormalities :

Lagerlof (1934), Anderson (1941), and Davis et al (1940 a) found lowered fertility when the semen of a bull showed more than 17 per cent abnormal spermatozoa.

Rollinson (1951) on the contrary could not find any close relation between abnormal forms and fertility in a study using 14 fertile and 29 sub-fertile bulls for natural mating. According to him semen sample having four per cent abnormal heads, 5 per cent detached heads, 10 per cent deformed midpieces and and 1 per cent abnormal tails showed a fertility of

60-80 per cent or 1.7 to 1.25 services per conception. Blom (1948) classified morphological abnormalities in two distinct categories (a) Primary due to defective spermatogenesis (im perfectly formed spermatozoa, small and possibly detached heads, double heads, double tails and immature forms) (b) Secondary as a result of degenerative changes following a normal differentiation of the spermatozoa (these included normal and detached heads, proximal and distal protoplasmic droplets, bent tails and detached galea capitis). According to him when more than 15 per cent of the spermatozoa showed primary abnormalities there is impaired fertility. Blom from his study reported 5 to 10 per cent primary and secondary abnormalities which appeared to vary. Cupps et al (1953) showed a strong correlation between abnormal forms and fertility.

Kaira district Cooperative Workers (1965-66) reported 13 to 14.7 per cent abnormal count in different seasons. Autumn being the best season with less abnormal count in buffalo semen. Bhosrekar (1975) also found similar result in Murrah buffalo bulls at NDRI Karnal, Hahn (1969) reported higher incidence of abnormal forms in older bulls. Rao (1971) also described a marked correlation between the age and abnormal forms of sperm head appearing in semen, Kodagali (1973) reported reduced fertility with increased abnormalities in buffalo bulls. Wells et al (1970) recorded a high proportion of abnormal forms in bull's semen when, bulls were rested for long time and again used. High percentage of acrosomal defects were also noticed by him. Subsequently regular collection improved the quality of semen. Bhosrekar and Razdan (1973) found that the tail abnormalities increased on low protein rations.

The main abnormalities can be categorised as follows

Head abnormalities	Mid piece abnormalities	Tail abnormalities	Other abnormalities
Narrow	Beaded	Simple tail bent	Proximal protoplasmic droplets
Narrow at base	Swollen	Coiled below head	Distil protoplasmic droplets
Pear shape	Broken	Coiled round head	-
Micro	Fibrils exposed	Broken neck	-
Giant	Abexial - attachment	'8' coil	-
Round	Cork Screw	Dag defect	
Loose normal	Double midpiece	Angular bent	-
Loose abnormal	-	Filliform tail	-
Double head	-	Double tails	-

Predisposition to functional disturbances : Weak sexual drive, disturbance in serving ability, disturbance in the mechanism of ejaculation, disturbance in spermiogenesis has been attributed by Nils Lagerlof to the weak endocrine constitution.

Somatic diseases : Optimum production of healthy normal spermatozoa is dependent upon health of the bull as a whole. Under the veterinary recommendation, the bulls must be routinely tested for presence of several diseases which affect the reproductive tract

of the bull or general health of the bull. No bull should be used in A.I. programme unless he is free from Campylobacteriosis, Brucellosis, Tuberculosis, Trichomoniasis, Johnes diseases, Leptospirosis, Listeriosis, IBR, IPV, etc.

Brucellosis causes inflammation of testicles (orchitis) epididymis (epididymitis). Epididymitis is also caused by several other infections like streptococci, C. pyogenes, E. Coli, Pseudomonas aeruginosa. Epididymitis changes the environment of epididymis for spermatozoa. It may reduce the motility of spermatozoa or create secondary abnormalities of tails eg. coiled tails.

Pathology of vasa deferens or ampulla is usually associated with an orchitis, epididymitis or seminal vesiculitis, the causative organisms may be brucella abortus, corynibacterium pyogenes or Pseudomonas aeuroginosa and viruses. Rectal palpation may reveal inflammed ampullae. Semen picture reveals leucocytes and infective organisms.

Seminal vesiculitis has been found affecting seminal vesicles of bulls, stallions and Boars. The incidence of seminal vesiculitis in bulls was reported as 0.8 per cent by Blom and Christensen in Denmark in 2000 bulls examined by them. 4.2 per cent by Vandershis in Holland in 825 infertile bulls and 4.6 per cent by McEntee in USA in 343 bulls examined.

Seminal vesiculitis may be caused by C. pyogenes. This may be sequel to primary pyogenic foci such as, liver abscesses, traumatic gastritis, lung abscesses or from naval infection in a yound calf (Galloway, 1964; McEntee, 1962). It may be possible to get the

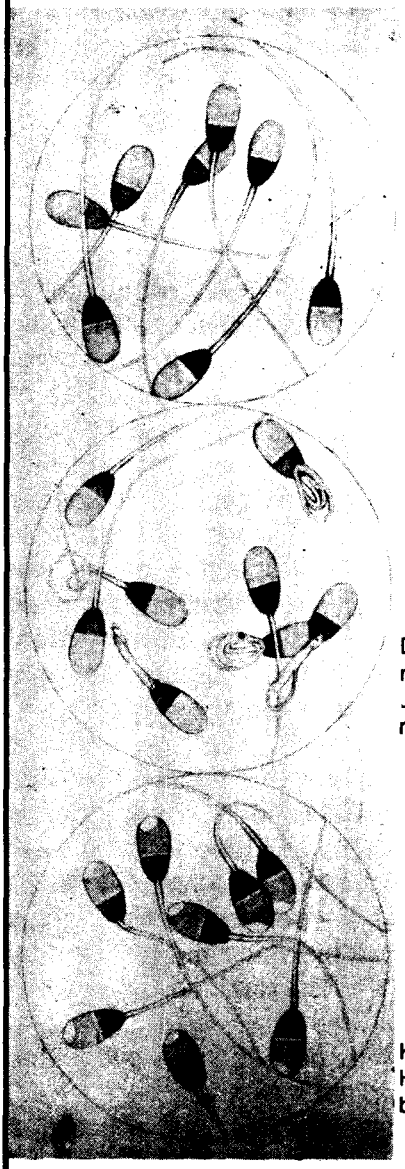
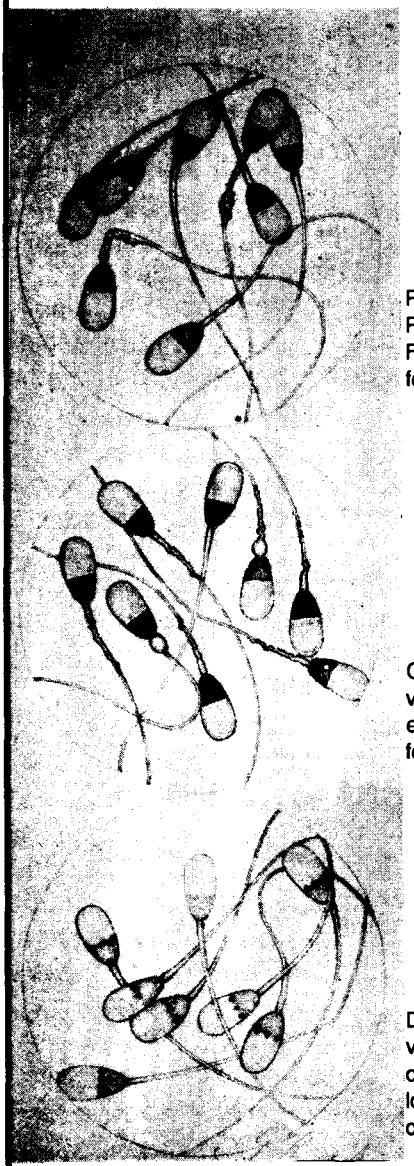


Fig. 24. Normal sperm.

Dag-defect. Hereditary, until now only found in bulls of Jersy breed. Lowered fertility, recovery not seen.

Knobbed-sperm defect. Hereditary, found in Friesian bulls. Total sterility.



Pseudodroplet defect. Probably hereditary, found in Friesian bulls. Lowered fertility.

Corkscrew-defect. Found in various breeds, mostly in elder bulls. Decreasing fertility, recovery very rare.

Diadem-defect. Found in various breeds. Indicator of disturbed spermiogenesis, lowered fertility. Recovery often seen.

infection by an ascending route from prepuce or by descending route from ampullitis, epididymitis or orchitis, Burcellosis is also common cause of seminal vesiculitis.

On rectal examination seminal vesicles are characterised by irregular enlargement, fibrosis peritoneal adhesions, loss of lobulations, fluctuations and abscess formation. In rare cases fistulas occur due to rupture of abscess in the recturn. Neutrophils appear in semen. In purulant type large clots or floculii were commonly observed while some times the semen is viscid or ropy. pH is elevated.

With high levels of broad spectrum antibiotics or the antibiotics to which the causative organisms are sensitive, the disease can be cured.

Disease of prostate gland is uncommon in all farm animals except dog.

Testicular tumors are unusual in most of the domestic animals except dogs and possibly old bulls. Interstitial cell tumors are benign type and are found in bulls at more than 7 to 10 years of age.

Testicular degeneration may be caused by febrile diseases temporarily. Sub-acute or acute trauma, stress cause rapid and progressive testicular degeneration.

Hoof troubles : Footrot, grown up hooves, interdegital condeloma cause painful conditions in bulls and inferlity coendi results.
Other pathological conditions affecting reproductive organs

Balano-posthitis : Balanitis is an inflammation of the glans penis and posthitis is an inflammation of the prepuce. They both are often involved in an inflammatory reaction because of their closer apposition. It is common in bulls, ram and dog but uncommon in boar and cat.

Trauma, abrasions, lacerations of prepuce or glans penis usually result in the introduction of the above wound infection, organisms enter into deeper tissues with resulting swelling, inflammation, pain etc.

Paraphimosis : This is the condition where bull is unable to withdraw the penis into the prepuce resulting in oedema, swelling and belanitis.

Phimosis : In this condition the bull is unable to protrude the penis thus the bull is unable to copulate or discharge semen.

Broken Penis : The condition of broken penis with secondary haematoma, occurs in bulls having strong sex desire and serves the cows in pasture. It is rarely observed in bulls which are hand mated.

Adhesions of retractors penis muscle : It is caused due to trauma and may prevent protrusion of penis and cause infertility in male.

Adhesions of glans penis : Adhesions of preputial and penile mucosa may take place causing difficulty in protrusion of penis.

Deviation of penis : This may be congenital and cause infertility because of inability of bulls to discharge semen in normal way.

Apparently campylobacteriosis (Vibrio foetus organisms) may not cause any trouble in bulls but it produces infertility in cows causing irregular repeated breeding and early embryonic mortality. Trichomonads present in the prepuce of bull, produce irritation and cause posthitis. These organisms (protozoa) can be seen under microscope in saline preputial washings.

Spastic paresis : Spastic paresis of hind legs is much commonly seen in inbred herds. The bulls cannot walk with normal gait and can not bear weight of their body and fall. Incidence is found in Danish Jersey and native Gir breeds. While selecting this aspect should be taken into consideration.

Feeding Faults (Nutritional factors)

Plane of Nutrition : Low plane of nutrition causes nutritional stress. As physical deterioration progresses into inanition in the male, there occurs an atrophy of the testis, a decrease in sperm concentration per ejaculate and progressive loss of libido. Delayed puberty will occur. The low plane of nutrition may adversely affect reproductive functions in the male. The severe effects on reproduction are only noticeable, unless emanciation and inanition is marked.

High plane of nutrition also some times cause infertility. High plane of nutrition can cause fattyness because of endocrine disfunction, though there is no evidence for this. High plane of nutrition did not show any disturbance in mating behaviour. Deakin and others (cited by Roberts 1971) indicated that excessively fatty bulls may have enough fat around testicles in the scrotum especially dorsal part. The fat causes insulation and affects spermatogenesis though this

has not been proved. Some excessive fatty beefy bulls are highly fertile. Excessive feeding or very high plane of feeding is not advisable but high energy intake at early age can be utilized for pushing growth rate in young bulls.

Proteins : Under the usual conditions of management the possibility of a deficiency in either quality or quantity of protein fed to bulls seems to be rare.

When the bulls are fed low protein ration less than 2 per cent with low feed intake, loss of weight, weakness and reduced libido and reduced sperm cell production occurred (Warnick et al 1961 and Meacham et al 1963). Urea was satisfactory as source of protein in ruminant male animals. There is no need of first class protein in ruminant diet like animal protein, skim milk, fish meal, turkey mash etc.

Bhosrekar et al (1986 a,b,c) fed crossbred bulls of 50% and 75% exotic Holestein Fresien inheritance with different levels of digestible crude protein and energy in a switchover design. They reported adverse effect of high energy ration. The primary sperm defects increased because of disturbed spermiogenesis on the contrary varying levels of digestible crude protein did not cause any significant effect on the quality of semen.

Vitamins : Amongst vitamins, vitamin A deficiency is very important from reproduction point of view. Avitaminosis-A, may cause cessation of spermatogenesis and atrophy of germinal epithelium and decline in semen quality as well as decrease in sexual desire. It also causes night blindness and stiffness. In young bulls it causes cystic pituitary

gland (Ghannam et al 1966; Erb et.al.1947). In ruminant the deficiency of vitamins B, C, D and E is not expected to occur.

Minerals : Mineral deficiency of calcium, sodium and potassium in large animals is not expected since enough of these minerals are available through feeds and fodders. Deficiency of trace elements like copper, cobalt, iron may cause anaemia, lack of appetite, loss of weight. The deficiency of trace elements like zinc, iodine and potassium have been theorised to cause infertility in male but has not been proved. High dietary calcium may cause spondylitis and arthritis.

Mostly gross under nutrition causes the reproductive disturbances in male but if the bulls are fed balanced ration and adequate quantities of trace minerals and vitamins, the growth recorded is optimum and reproduction is normal. High protein in adult bulls may cause higher amount of sperm abnormalities and stimulates bulls for masturbation because of preputial irritation (Hultnas, 1961).

Environmental factors : Climate is a combination of several elements, such as temperature, humidity precipitation, air movement, radiative conditions, barometric pressure and ionization. Animal husbandry in tropics is affected by climate in two ways, first by the direct influence on the animal and secondly by the indirect effect on the animals' environment.

High air temperatures have some effect on the fertility of bulls though reproductive cycles of the cow do not seem to be affected unduly. High air temperatures have more adverse effect on the bulls of temperate breeds rather than tropical breeds. Experimental

evidence has shown that high testicular temperature affect the spermatogenesis, increase sperm abnormalities and reduce sperm concentration. High environmental temperatures have been shown to affect sex desire of bulls and the bulls loose libido. At N.D.R.I. during summer the author has observed not only loss of libido in imported exotic bulls of Brownswiss and Friesian but the ejaculates donated by them were found to be watery and with low sperm concentration. This was not true with Jersey to the same extent as Brownswiss and Friesians. It has also been reported recently by researchers in America that due to introduction of deep freezing of semen it has been possible to test the real fault, whether it lies with semen of bull or female reproductive efficiency and it has been reported that frozen semen from summer ejaculates used in the best part of the year on normal (having good reproductive efficiency) cows have poor conception rate as compared to frozen semen from ejaculates collected during other parts of the year. It has been shown by Bhosrekar (1975) that buffalo bulls donated best quality of semen during monsoon followed by summer. Winter was found to be the worst season for semen production. Early morning semen collections and splashing of water on the body of buffalo bulls twice daily during summer was recommended by him .

Bucks lost complete libido during the months of May and June at N.D.R.I. because of high air temperatures but the libido was regained during months followed subsequently because of reduction in air temperature. The maximum air temperature noted at Karnal was 42° C and for the months of May and June the range is 38° to 40° C.

Managemental Factors : Bulls vary in their active life from 5 years to 16 years. The age of the bull has no influence on the quality of offsprings produced. The exotic bulls in temperate zones are ready at 12 months for donating semen but the same bulls if brought up in tropical area will not be ready to donate semen before 18 months, while indigenous bulls of different breeds are ready for semen donation at 2 to 2 .5 years of age. The temperate breeds will donate semen until six years of age but tropical breeds will not decline semen donation so early and will continue to donate for fairly longer periods. Buffaloes donate semen at 2.5 to 3 years of age and continue for more than 11 to 12 years of age without any problem.

Frequency of services : Young bulls can be used once a week till 2 years of age, while bulls above 2 years can be safely used twice a week until six to seven years of age. However, older bulls should be used at longer intervals. Maximum semen can be collected and frozen in the prime youth of the bull as the quality gets deteriorated as the age advances. Therefore, it is advisable to use bulls between 15 months to 3 years to the maximum possible extent and the bulls can be disposed off.

Housing : Well ventilated, well insulated houses should be provided to exotic bulls. The floor should be cemented (pucca) with soft bedding. Half of the floor area should be loose sandy. Well built wide feeding manager and water trough should be provided. The bull should be left free in individual bull pen.

Cleanliness should be strictly observed. In regions where there is very high temperature like North India and Middle India specially in summer and high humidity

like East and West regions during the summer, ceiling fans should be provided. The hot wind blasts should be cut down by providing curtains, (Khas curtains are most comfortable). For one bull a floor space of 10' x 15' should be provided with shed and similar area uncovered. The roof should be at 12' height and should be either of mangalore tiles or asbestos sheets or cemented. Adequate clean and cool water should be provided for drinking. The floor should not remain damp or wet and good drainage should be provided. Damp floors predispose the bulls for soft hooves. For tropical breed bulls similar type of housing should be provided. Fans may not be required since they are well adapted to the climatic conditions.

Feeding : The young bulls should be liberally fed in order to get maximum growth and early maturity. Water and salt lick should remain all the time before the calf. As soon as the bulls become 16 to 20 months, the rations should not be liberal any more and the bull should be given balanced ration and should not be over fed. For good fertility the bull should be kept fairly thin. High fat containing ration should not be fed to bulls. A mixture of barley, oats, wheat bran and groundnut cake can from good concentrate with 40 to 50 kg. of green fodder and 2 kg of dry fodder.

Exercise : Bulls should be exercised on mechanical exerciser in a lot for one to two hours. This is required where loose housing is not followed. Bhosrekar and Nagpaul (1973) reported beneficial effect of exercise for getting good quality semen from bulls. It also reduced reaction time in indigenous bulls as the exercise kept the bulls in good muscular condition and is very important in obtaining maximum quantities

of good fertile semen. Herman and Madden 1953; reported that exercise helps stalled bulls in improving semen quality.

Transportation : Transportation of bulls cause lot of stress on the animal and affects the quality of semen due to the environmental changes and the surrounding changes. There is thus psychic factor involved in. Some times bulls refuse to donate the semen. This has been observed by the author in buffalo bulls. Buffalo bulls were transported for buffalo project of NDRI Karnal from different parts of India. It was observed that, some of the adult bulls which have donated semen at the home place never donated the semen on transportation to NDRI, Karnal. Out of ten bulls 3 bulls were found to be of this type.

Psychic factors : Some bulls without any apparent clinical symptoms hesitate to mount and donate semen. Such bulls suffer from weak endocrine constitution and little disturbance will cause nervousness. Little switching of tail by stimulus animal or its movement will also cause hesitation in the bull to mount.

If two or more than two buffalo bulls are brought at a time they will not donate semen but always be afraid of each other. The site of collection should be away from public, and the strangers should not be allowed at the site of collection as the bulls get nervous from the sight of strangers. The semen collector and his dress should not be changed often. The attendants also should not be changed. All these factors cause psychic troubles in bulls especially those with lowered sexual desire. Bulls suffering from spondylitis or arthritis or traumatic recticulitis feel pain in mounting,

so hesitate even though the bulls have good libido. The low roof also cause nervousness in bulls as they are afraid of colloiding with the roof.

The site of collection, the preparation of bull like washing cleaning of sheath, wiping with towel, tying of apron, all play as conditioned reflexes, and these help in stimulating bull properly for donation of semen.

Grooming of the bulls, cleanliness of the prepuce are very essential for good quality semen. The grooming of bulls will remove the loose hair from the body. The bulls should be washed daily. The dung sticking to the body especially to lower part of the bull like sheath abdomen should be removed. Use of bull aprons should be encouraged.

CHAPTER XIII

REPRODUCTIVE EFFICIENCY AND ITS MEASURE

Biometry of testis : Willett and Ohms (1957) Altman and Dittmar (1962) and Hahn et al (1969) have reported a close relationship between scrotal circumference and volume of water displaced by the testis in a measuring bucket and the spermatozoa produced in exhaustion trials in bulls. There is no relation between body size, scrotal size and the number of spermatozoa in a single ejaculate. Bhosrekar et al (1980) reported highly positive significant correlation between body weight, heart girth, length, height at witheres. However, they could not record any correlation between scrotal volume and sperm concentration or ejaculate volume. Thibier (1972) also reported similar finding. The average scrotal circumference in mature bull is 40 to 42 cm. with a range of 37 cm to 46 cm. The testes is 10 cm to 13 cm long and 5 cm to 6.5 cm wide at maximum width and weigh 500 g. It is covered by a thin layer of elastic connective tissue, the tunica albuginea. On palpation the testes is turgid and firm.

The beginning of the active spermatozoa production as evidenced by the presence of mature spermatozoa in the testes was at 7 1/2 to 8 1/2 months of age (Phillips and Andrews, 1936; Hooker 1944). Baker and Van Demark (1952) reported 29 weeks as the age of maturity for Holstein Friesian bulls. They have shown rapid development of penis from the time of first interest in sex to first ejaculation. The glans penis is cleared of the preputial adhesions.

Hahn et. al. (1969) developed a tonometer and measured the consistancy of the testis. This was highly correlated with the semen quality, a soft or very firm consistancy was often related to poor semen quality.

Highly significant correlations are found between scrotal circumference with body measurements, age and semen production by Kupferschmeid et.al.(1975); Tierney et.al. (1982); Jakubiee (1984) Salam Daudu and Shoyinka (1983); Heuston et.al. (1988).

The relationship between semen characteristics and fertility : Beck and Salisbury (1943), Van Demark et al (1941) in an attempt to establish relation between semen evaluation and fertility found negative significant correlation between M.B.R.T. and fertility. Sperm concentration and fertility had positive significant correlation. The measures of fertility are non-return 60-90 days.

Recently certain biochemical parameters have been found to bear high relationship with fertility of semen. (Such as Glutamic oxalo acetic transaminase, Acrosin and Hyaluronidase enzyme activity). Appearance of the activity in seminal plasma will indicate the damage to the mitochondrial sheath of spermatozoa or acrosomal cap of spermatozoa (as these enzymes are essentially intracellular). This directly reflects on the fertility of semen.

Measure of fertility : The measure of fertility should be based on number of first services and actual conceptions as diagnosed by rectal palpation since in India non-return rate has no meaning. Farmer is not careful to watch the cows or buffaloes for appearance

of subsequent heats and once inseminated he thinks that the cow/buffalo must be pregnant. Secondly anoestrus because of poor nutritional status of cows may also lead to wrong interpretations.

$$\text{R.E.} = \frac{\text{No. of Conceptions}}{\text{Nos. of 1st services}}$$

Bhosrekar (1975) has found a difference of 30 to 40 per cent in actual conception rate and 60-90 days non-return rate in buffaloes while a difference of 6.7 per cent existed between actual pregnancies and 60-90 days non-return in cows.

Staining procedures for estimations of sperm abnormalities :

Preparation of semen smears : Care must be taken to prevent cold shock during and after collection and until sperm cells are fixed on the microscopic slides. Cold shock will cause coiled tails and may lead to erroneous finding. As the population of sperm cells is very ligh, taking a drop of fresh semen for preparing smear will cause difficulty in examining morphology of individual sperm as it is likely that sperm will overlap each other. Therefore, semen should be diluted in proportion of 1:10 in isotonic diluent (e.g. physiological saline, ringer's solution or isotonic solutions of citrate or phosphate buffers). The temperature of the diluent and semen should be the same at the time of dilution. Mixing be done in small culture tubes.

After mixing a small drop of diluted semen should be placed on a clean, grease free microscopic slide.

Another clean slide is placed base down at an angle of 30 degrees with the first slide, and the drop is allowed to flow along the apex of the two slides. The second slide still held at the same angle is pulled gently along the first to spread the drop. Excessive pressure should not be applied, the smeared slide should be allowed to dry in a flat position.

Before staining the slide should be immersed in alcohol for 2 to 5 minutes for fixing.

staining smears of semen for acrosome morphology : The acrosome and its contents appear to be involved in the penetration of the spermatozoa through the corona radiata and zona pellucida of the egg (Bedford, 1972). Disintegration of acrosome of the spermatozoan as a result of chilling and deep freezing might therefore be expected to affect its fertilising capacity.

A drop of diluted semen is smeared on a prewarmed slide and dried in a current of warm air. The smears are fixed by immersion in a buffered formal saline (Campbell and others, 1960) for 15 minutes and washed in running tap water for 15 to 20 minutes. The smears are dried and then immersed in buffered Giemsa stain.

Preparation of stock solution of Giemsa : Giemsa stain 3.8 g was ground with absolute methanol (AR grade 375 ml) in a pestle and mortar, Glycerol (125 ml AR grade) was added and the stain mixture was stored at 37°C for one week. During this period it was shaken for a few minutes each day.

Preparation of staining mixture : 3.0 ml. of the stock solution diluted with sorenson's M/15 phosphate buffer (pH 7.0) 2.0 ml. and distilled water 35 ml.

By means of this simple, rapid technique, involving examination of fixed, stained specimen with light microscopy, the integrity of acrosomes can be assessed. The stain can be used routinely for staining bull, ram, buck semen and components of various diluent mixture, e.g. egg yolk, glycerol etc. do not show any marked influence on the quality of staining. In all cases highly satisfactory differential staining of acrosome is seen.

The stained sears should be examined after 90 minutes of staining washing and drying.This method is by Watson (1975).

Vital Staining for estimation of live sperm cells : For estimating live sperm cells in a sample of semen, prepare diluted semen 1:10 in isotonic citrate, phosphate or Tris buffer.

Prepare stain as follows :

Eosin (blue) (GURR) Powder	100 mg
Nigrosin powder	500 mg
Sodium citrate buffer (2.9% solution)	10 ml

The stain should be prepared fresh every 10 to 15 days. For preparation of stained smears take 10 drops of warm Nigrosin-Eosin stain in 2 ml. tube add to it one drop of diluted semen (1:10). Mix it properly by rotating in palm. Take a drop of this mixture on a warm slide and prepare a smear as described earlier.

While mixing, the stain and semen should have the same temperature otherwise all abnormalities may appear because of thermal shock. The live sperm will not stain while dead sperm will take stain as on death the cell wall of dead sperm becomes more permeable to stain. The same smear can also be utilised for assessing sperm abnormalities.

Reliable estimates of abnormal sperm cells in a semen sample may be made by counting more than 100 cells in a single smear.

For all practical purposes 200 cells in 20 different fields gives most satisfactory results.

Giemsa stain was first introduced by Hancock (1952) for studying the acrosomes. Giemsa stain is known to be sensitive to the change in H-ion concentration. Therefore, the stain should be properly buffered before the application.

CHAPTER XIV

OESTRUS SYNCHRONISATION

Detection of heat in dairy cattle and buffaloes is time consuming and often difficult job. Regulation of oestrus cycle therefore, where by most of the cattle and buffaloes in the herd could be brought into heat one time would be highly desirable from practical and economic point of view.

Cattle owners, Farm managers and veterinarians would welcome a technology whereby cows and buffaloes could be inseminated on predetermined date and at a specified time without need to observe oestrus behaviour. Ideally speaking such a technology should be nontoxic with little or no side effects to the animal and the conception rate on treatment should be optimum.

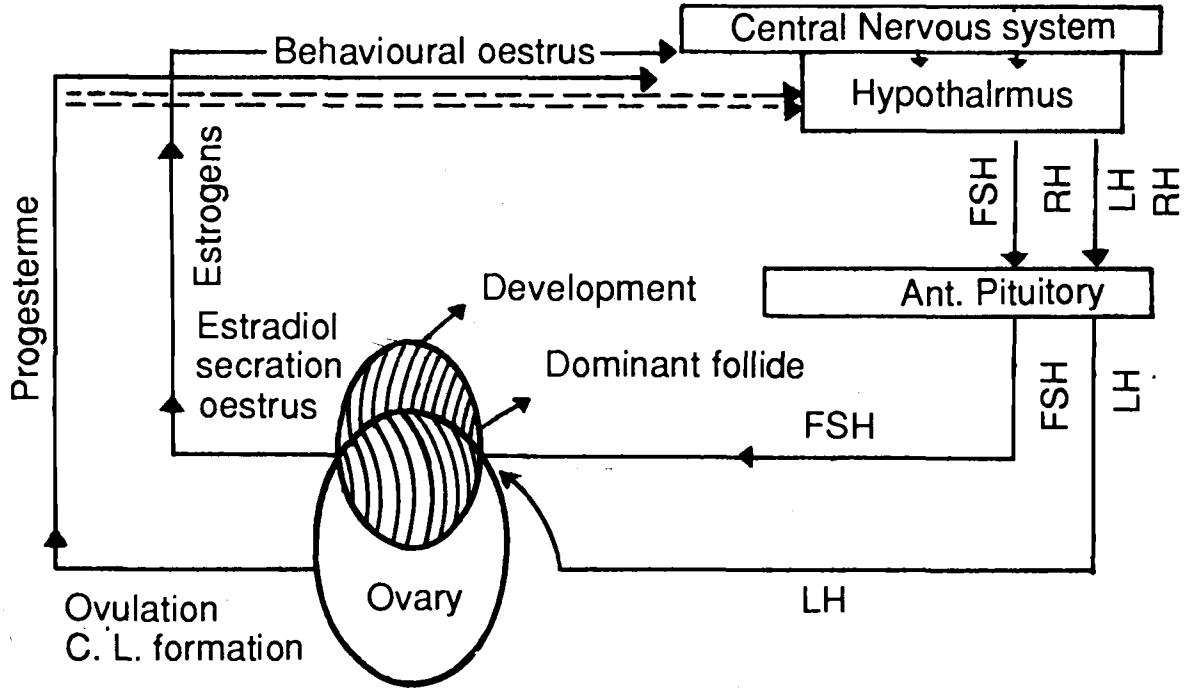
Advantages of Synchronisation of oestrus :

Inseminations could be done on pre-determined date and time which becomes more economic and helps in efficient management.

Planned breeding programme would facilitate introduction of genetically superior sires and would lead to predictable shorter and compact calving periods.

Regulation of milk market and management of rearing calves as well as marketing becomes easier.

Seasonality in caving can be broken and milk can be made available all round the year.


Central Nervous system
Behavioural oestrus
Hypothalrmus
FSH
RH
LH
RH
Estrogens
Progesterme
Development
Ant. Pituitory
Estradiol
secration
oestrus
Dominant follide
FSH
FSH
LH
Ovary
Ovulation
C. L. formation
LH

It helps in getting calf crop simultaneously from different bulls which will help in early progeny testing of the bull.
Problem of silent heat will be overcome.

Physiology of oestrus cycle :

Before going into details of synchronisation techniques it will be appropriate at this stage to understand the physiology of oestrus cycle.

Definite physiological functional rhythms of reproductive system in animals that reached puberty is called as oestrus cycle. There are two distinct phases of the cycle. 1. Oestrogenic or follicular phase. 2. Progestogenic or luteal phase.

In the first phase graffian follicles in the ovary start maturing, become dominant and one of these dominant follicles goes to the surface of the ovary and ovulates while others get degenerated. All this chain of action takes place under the influence of follicular stimulating hormone (FSH) and luteinising hormone (LH) in that order. These gonadotrophins are secreted and released from anterior pituitory by the action of FSH-RH and LH-RH combindly known as Gonadotrophin Releasing hormone (GnRH) coming from Hypothalamus. (Fig. 24)

The dominant graffian follicle secretes oestrogens from the theca interna cells and behavioural expression of oestrus in cows is seen. The oestrus in cow/heifer remains for 18 to 24 hours. Much before the rupture of graffian follicle (ovulation) the cow/heifer ceases to be in heat (11 to 12 hours). On ovulation the cow/heifer goes in progestational phase.

The ovulation takes place because of increased release of LH and lowering the release of FSH from the anterior pituitory. It has been observed that LH surge comes about 24 hours before the ovulation. The luteal cells start organising at the place of rupture of follicle and a body red in colour in the begining is formed (Corpus rubrum). This body gets changed soon to yellow colour and remains in the ovary till 18th day of the cycle. This body is known as Corpus luteum, and secretes actively a hormone called progesterone. Under the influence of this hormone the animal goes into quiescent state (Diestrus).

It has been observed that concentration of estradiol in jugular vein increases from 2 to 4 pg/ml to 10 to 20 pg/ml at least three different times during oestrus cycle. Ist, during oestrus, 2nd after ovulation in early diestrus (on day 5th to 6th) and 3rdly, during 10th to 16th day of the cycle before luteal regression.

Since estradiol is produced by the ovarian follicles and not by the luteal tissue, in cattle, therefore, three rather than two waves of follicular growth are likely to occur coincident with each of these sustained increases in estradiol.

According to Ireland and Roche (1983 a,b) a single estradiol active follicle several mm. larger than the next largest follicle is present during oestrus, early dioestrus and mid dioestrus although one or two other follicles > 6 mm in diameter also exist on ovarian surface but subsequently get attretic and have higher content of progesterone and androgen in follicular fluid. According to these authors cattle have dominant ovulatory and dominant nonovulatory follicle. Each cycle of development of follicles goes through a selection, dominance and attresia or ovulation phase.

In selection phase many follicles begin to develop but undergo attresia before a single follicle emerges as a dominant follicle. At the end of the selection phase a single follicle becomes larger than all other follicles and is responsible for most of the estradiol production. This production of estradiol by a dominant follicle contributes to a trasient increase of estradiol in peripheral circulation. If regression of corpus luteum does not occur during a dominant phase this follicle undergoes attresia and a new selection phase begins.

Although the precise role of estradiol during each of the cycle of development of dominant follicle is not known, yet each sustained rise in estradiol level coincides with an important physiological event.

The rise in estradiol blood level between day 10 to 17 precedes luteolysis. Since estradiol is luteolytic in cattle, dominant non ovulatory follicle may have a role in initiation of luteolysis. The rise in estradiol during luteal regression through oestrus is likely involved in initiation of preovulatory LH surge in cattle and occurance of behavioural oestrus.

The sustained rise in estradiol in the begining of the diestrus (lutealphase) between day 5th to 6th after ovulation coincides with the time, a blastocyst enters the uterus in cattle. Indeed dominant ovulatory and non ovulatory follicles may be involved in control of cascade of events that take place during bovine oestrus cycle which lead to a successful pregnancy.

The individual follicle continues to develop during its dominance phase and simultaneously blocks development of other follicles. This process is very important from the point of view of regulation of

follicular development. Inhibin, another hormone secreted by granulosa cells of graffian follicle is present in follicular fluid. During dominance phase, a dominant follicle produces an increased amounts of inhibin, follicle regulatory protein (FRP) and estradiol. Inhibin acts on the pituitory gland to lower FSH secretion and thus blocks recruitment of new dominant follicles. FRP from dominant follicle blocks synthesis of estradiol in the non dominant follicles remaining after the selection phase which results in attresia of non dominant follicles. Estradiol in the dominant follicles increases the responsiveness of the follicle to gonadotrophins present in the blood, thus ensuring continuous development of follicle till ovulation, however if ovulation doesnot take place levels of FRP increase in the dominant follicle thus causing reduction in estradiol production and increase in progesterone and androgens in follicular fluid resulting in attresia of dominant follicle. Since levels of FRP, inhibin and estradiol would decrease after ovulation or attresia of dominant follicle, a new cycle of development of dominant follicles begin by increase in blood level of FSH.

A higher concentration of vitamin A in non attretic dominant follicles has been observed by Ireland et al (1985) which would, therefore, mean that maturation of oocyte and development of follicle specifically require an increase in vitamin A supply.

In normal course of events if pregnancy doesnot take place the corpus luteum gets regressed on 18th day of cycle because of preceding higher estradiol level and naturally occuring prostaglandin F2 α in uterine secretions. This sharp decline in progesterone blood level triggers release of FSH by anterior pituitory

under the influence of GnRH (FSH-RH) from Hypothalamus and a new cycle sets in.

Taking advantage of this naturally occuring events, oestrus synchronisation can be carried out by exogenous administration of progesterone or its synthetic analogue and extending the progestational phase. Removing the source of progesterone will thus trigger hypothalamus to release FSH-RH resulting in release of FSH from anterior pituitory and stimulating growth of follicles in ovary bringing the animals in heat.

Porgesterone hormone is available as its synthetic analogue called as 'progestogens' which are potent to mimic the action of natural progesterone.

Methods : The administration of progestogen can be done by way of feeding, daily intramuscular injections or inserting long acting depot preparations under the skin or in Vagina of the cow.

The commonly available progesterone preparations are as follows with their dosages.

1. MAP (Methyl acetoxy progesterone) which is less potent and requires a dose of 180 to 200 mg per day through feed.

2. CAP (6 chlor - 6 dehydro 17 - acetoxy progesterone) or chlormedinone where a dose of 10 mg/day is recommended.

3. MGA (Melengesterol acetate) a highly potent progestogen preparation. Only 1 mg/day is quite sufficient to produce the desired effect.

All these above preparations are meant for oral administration, such treatments need long period of 14 to 18 days. It is more cumbersome and tiring.

Progesterone also can be administered by daily intra muscular injection of 25 mg for 10 days for desired effect. In this method daily visit to animal makes it uneconomic and tiresome.

Recent advancements in this biotechnology has solved this problem. A depot source of progestogen by way of subcutaneous implants or intravaginal sponges or silastic coils made this technology much easier.

PRID : (Progestogen releasing intra vaginal device). These are silastic coils impregnated with 1.55 g progestogen and has a gelation capsule of 10 mg estradiol benzoate attached on the inner surface. This provides a source of progesterone and estradiol which gets absorbed through vaginal mucus membrane slowly. This coil is kept in the anterior part of the vagina of cow for 10 days and is removed. On removal of this coil source of progesterone is withdrawn, causing a sharp decline in blood level of progesterone thus starting the sequential events and bringing the cows in heat. The cows could be inseminated at 48 and 72 hours after the removal of silastic coil from vagina.

A combined treatment for better and assured results is very popular. In this, PRID (Silastic coil) is inserted in the vagina. On 8th day after insertion of PRID. 25 mg of PGF2 α and 400 IU of PMSG (Pregnant mare serum Gonadotrophin) is given intra muscularly. On 10th day the PRID is removed and on 12th and 13th

day the cows are artificially inseminated. 100% occurance of oestrus and 55 to 62% conception rate has been reported in cows and heifers.
(Lokhande et al 1983, 1984, and Bhosrekar et.al. 1986).

Implant : A subcutaneous implant containing 6 mg of norgestomet (17 acetoxy 11 ß - methyl-19-nor pregn-4. One 3, 20 dione) is placed by special syrince subcutaneously on the outer portion of the ear for 10 days. At the time of implanting 6 mg of estradiol valerate and 3 mg of norgestomet are injected intramuscularly. On day 10th the implant is removed. The cows/heifers are inseminated on 12th and 13th day as in case of PRID.

Combined treatment also can be successfully used in case of implants, injecting 25 mg of $PGF_2\alpha$ and 400IU of PMSG on 8th day of insertion of implants as per PRID. With this treatment 100% cows and heifers come in heat. Intensity of heat is excellent and conception rate to the tune of 52 to 55% is obtained. (Lokhande et.al.1983, 1984, and Bhosrekar et.al 1986).

Prostaglandins : The second method of oestrus synchronisation is by use of luteolytic agent to cause pre mature regression of corpus luteum thus setting the sequence of events to happen for bringing animals in heat.

More recently a series of compounds have been examined which are termed as prostaglandins. These compounds have shown luteolytic effect and satisfactory post treatment fertility.

Prostaglandins are naturally occuring in the tissues and is also produced by uterine mucosa. It is responsible for causing lysis of corpus luteum in non pregnant cycling cows/heifers and bringing the animals in heat.

A sharp decline in progesterone level following the administration of PGF 2 α is similar to that of following normal regression of corpus luteum on day 18th of the cycle. These prostaglandins are not effective on corpora lutea below 5th day of the cycle.

In random group of cows/heifers there will be individuals at different stages of oestrus cycle and as a result, a fixed time insemination after injection will not be equally appropriate for all of time.

A lower oestrus response and lower fertility was observed in dairy heifers injected with PGF 2 α during early diestrus than in heifers injected after 10th day of their cycle (Watts and FUQuay, 1982, 1983).

The work of Ireland and Roche (1983 a, b) and Ireland et al (1985) showed that a single estradiol active graffian follicle several mm. larger than next largest follicle is present during oestrus, early diestrus and mid diestrus which suggests that currently used 11 day interval between 2, PGF 2 α injection regime may not be optimal. An 11 day interval between injections means that cows with responsive corpora lutea at first injection (about 60% of the randomly cyclical group) will be at about day 8 of the new cycle for second injection where as those which have immature corporalutea will be at day 8 to 11. By extending the interval between injections of PGF2 α to 14 days, the second injection would be made on day 11 instead of

8 or day 11 to 14 instead of day 8 to 11 of the new cycle. Thus all cows would be brought into the period after estrus which would produce optimum conception rates.

The longer period between injections would also accomodate the small proportion of females that appear to have delayed oestrus or a short cycle after the first injection.

The synthetic analogue available in the market is marketed under the trade name of Estrumate, synchromate, chloprostenol, ICI 80996 from ICI and Searl companies.

The natural PGF 2 α is marketed under the trade name of 'lutalase' Dinoprost, Dinafertin resectivaly by Hoechst, UpJohn and Alvid India companies.

The protocol for oestrus synchronisation using PGF 2 α will be then : Two injections with the interval of 14 days and on 72 and 96 hours of last injection artificial insemination to be done. Young (1989) obtained 51% conception rate with this protocol as compared to 44% with 11 day interval protocol.

Oestradiol also has the property of luteolysis but the fertility post treatment is not satisfactory. GnRH (Receptal, Hoechst) has been successfully used for induction of oestrus in cows, treatment of repeat breeders, because of delayed ovulation or anovulatory heats.(Bhosrekar et.al. 1986).

Limitations for the use of this biotechnology : The cows or heifers to be involved for oestrus synchronisation should be healthy, free from systemic

or reproductive disorders. Cows suffering from metritis, endometritis, cervicitis, salphingitis, impatent salphinx, cystic ovaries should not find place in the oestrus synchronisation programme.

Debilitated or unthrifty animals donot show desired results.

The cows/heifers should be cyclical and therefore all cows to be included in this programme should be screened for cyclicity.

CHAPTER XV

BREEDING MANAGEMENT FOR BETTER FERTILITY

This subject has four aspects, and these four aspects are like four legs of the table. Even if one leg fails to support the table, the table tumbles down. These four important aspects are :

1. Cow/Buffalo itself
2. Cow/Buffalo owner or farmer
3. Inseminator
4. Semen.

1.0 Cow/Buffalo : The general and reproductive health of the cow/buffalo has to be perfectly sound for proper breeding. She should be cycling and should not have anatomical, physiological and genetical defects. She should be free from reproductive disease or inflammation of reproductive tract.

1.1 Anatomical factors causing reproductive disorders:Congenital (inborn) defects like double cervix, white heifer disease, persistent hymen etc. may interfere in the proper breeding of the animal.

1.2 Genetical factors causing reproductive disorders These disorders are hereditary in nature. Single autosomal recessive gene is responsible for causing these disorders. These include hypoplasia of ovaries, (under development). In this condition one or both the ovaries are small, narrow and functionless. Lagerlof (1939) found an incidence of 1.3%. The affected cows had 87% hypoplasia of left ovaries, 4.3% of the right and 8.6% of both ovaries. Where both ovaries

were affected the cows were infertile and oestrus cycle did not occur. By the adoption of a vigorous control programme by veterinary examination of breeding cattle and culling of those having unilateral or bilateral hypoplastic ovaries the incidence of hypoplasia was reduced (Lagerlof and Boyd 1953).

Segmental aplasia of mullerian duct : These are the developmental defects of mullerian duct leading to various anomalies of the vagina, cervix and uterus. The affected animals show normal cycle, behaviour with usual secretory activity of the tubular genital tract. Uterus unicornis, absence of one salphinx, absence of cervix are some of the examples which lead to fertility failure.

Lethal factors and sublethal factors : Lethal factors are those factors where the conceptus grows to full gestation period but can not survive outside on delivery, or cause death of conceptus at developmental stage. Those which have been carefully studied are the one which result in late fetal death or in some deficiency preventing survival after birth. Gilmore (1952) has presented the list of lethals in cattle. Viz Achondroplasia of four types amputated calves, congenital dropsy, hairless calves, skinless calves etc. These are of genetical origin. Recessive genes are responsible which get exposed in the homozygous conditions.

All the conditions caused by recessive genes can be overcome by adopting sound breeding practices as they get chance to express themselves only when close inbreeding is practised.

Normally 33% of the breeding bulls are advised to be replaced every year at bull station to avoid chances of inbreeding but with the advent of frozen semen technology, the frozen semen remains in stock for longer time even if the bulls are culled. Therefore there is a potent danger of inbreeding in artificial insemination with frozen semen if breeding programme is not carefully monitored. Great care needs to be exercised in formulating and monitoring breeding policy while using frozen semen for A.I.

1.3 Physiological or functional disorders : Most functional aberrations occur because of some endocrinological abnormality which is frequently difficult to specify even with the current methods of hormone assay. The abnormalities occur as a result of weak endocrine makeup; nutritional deficiencies or excesses, social influences which may arise from modern husbandry methods eg. grouping of large number of cows, thus interfering with establishment of a stable social heirarchy; and the stress of production. The importance of functional forms of infertility has been shown in a survey of 283 herds over a period of 8 years in Finish Ayrshires (Roine and Sonolini, 1978) cystic ovarian disease was the most common cause of infertility occuring in 37.1% of infertility cases followed by subestrus (20.9%) anoestrus (13.5%) anovulations and delayed ovulations (5.6%).

1. When the ovaries remain quiescent or inactive the condition is referred to as anoestrus.
2. When the cow is showing normal cyclical ovarian activity but behavioural signs are not normal, this condition is described as subestrus.

3. The corpus luteum remains on the ovary exerting inhibitory effect upon the anterior pituitory. This may be persistent corpus luteum or cystic corpus luteum.

1.3.1 Anoestrus :
When this occurs the ovaries are quiescent with an absence of cyclical activity. The reasons may be insufficient release or production of gonadotrophins to cause folliculogenesis, or it may reflect the failure of ovaries to respond.

Some cows resume cyclical ovarian activity within few days of calving and then become anoestrus. In a study involving 535 dairy cows in four commercial herds Bulman and Lamming (1978) found that 5.2% of the cows showed this pattern of activity.

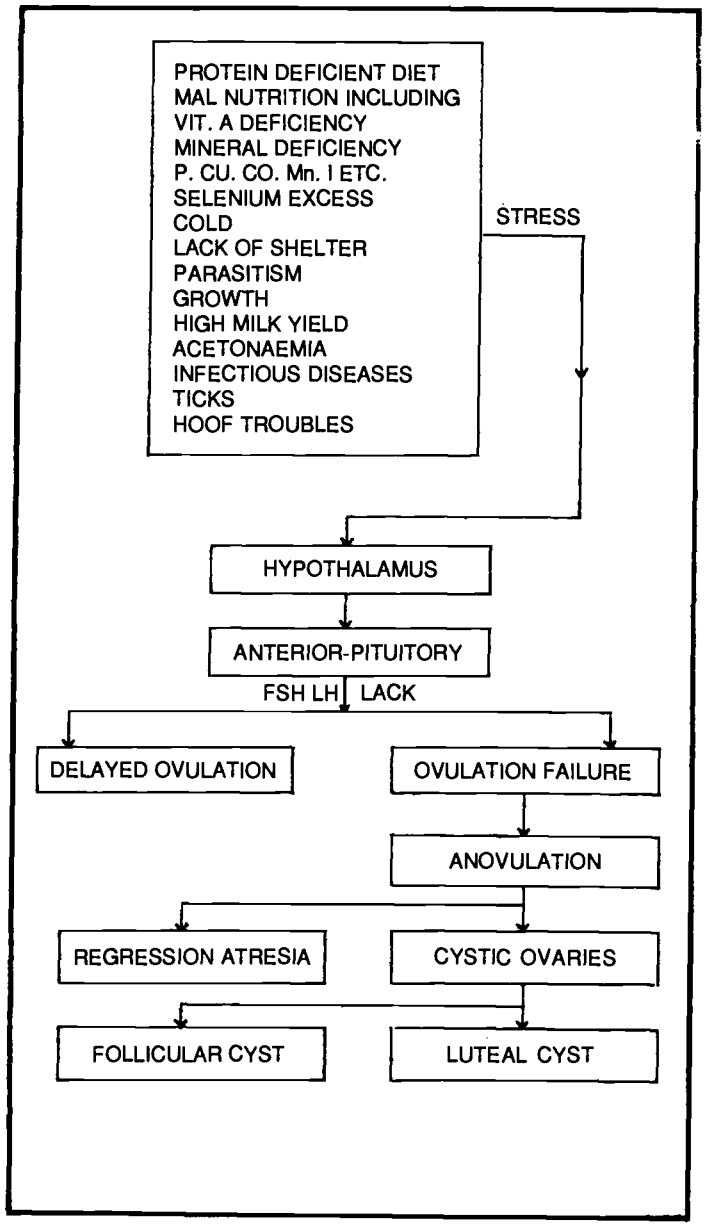
True anoestrus is most frequently diagnosed in high yielding dairy cows, first calf heifers and suckler cows. Suckling has a profound effect. (Lamming, 1980). Anterior pituitory appears to be refractory to stimulation with GnRH in the immediate post partum period. The refractory period is probably due to the duration of progesterone induced negative feed back (Lamming 1979). If prolactin secretion is high, prolactin inhibitory factor (PIF), secretion by hypothalamus is low and this also suppresses GnRH secretion and hence the production of gonadotrophins (Hafez, 1975).

Improving feeding particularly energy intake may be able to bring those cows back in cycle. PMSG can be used to stimulate or induce ovarian activity in a dose of 3000-4500 IU. GnRH has been used successfully to treat anoestrus in a single dose of 0.5 mg. Progesterone with oestrogen has been used to induce ovarian post partum activity (Foote and Hunter 1964;

Britt et al 1974; Wischort & Young, 1974). These are effective because they either stimulate the short luteal phase that usually precedes the first normal oestrus cycle. (Lamming, 1980). PRID, (See chapter XIV) which is easily inserted and readily removed when put in anoestrus cows for 10 days brings most of the cows in oestrus. 1% Lugal's iodine (20 ml) can be infused in the cervix or painting of cervix with lugal's iodine also has been found to be beneficial. Clomophine citrate (Fertivet 300) has been reported to be beneficial in inducing oestrus in cattle and buffaloes. (Deshpande et al, 1976; Pendse et al 1977; Kaikini et al 1977; Manjunath, 1979; Pillai 1980; Kurien and Mahadevan 1985)

1.3.2 Subestrus : A number of authors (Casida and Wisnicky, 1950; Morrow etal, 1960; King etal, 1976) have shown that the first and second ovulations post partum are frequently not preceded by behavioural signs of oestrus and are thus truly "silent heats". After the second oestrus it is unlikely that many true silent heats occur. Hall et al (1959) reported an incidence of 10.6% of silent heats even when cows were examined four times in 24 hours. Bhosrekar and Bhatnagar (1971) reported 5 to 7% incidence of silent heat in Indigenous milch breeds of cows Sahiwal, Tharparkar, Redsindhi.

A genetic predisposition to silent heat has been identified (Labhsetwar et al, 1963) with certain sire lines showing a statistically significant effect. A prostaglandin $F_2\alpha$ treatment followed by fixed time insemination is indicated. Prostaglandin $F_2\alpha$ infusion intra cervical also helps to intensify heat symptoms.


PROTEIN DEFICIENT DIET
MAL NUTRITION INCLUDING
VIT. A DEFICIENCY
MINERAL DEFICIENCY
P. CU. CO. Mn. I ETC.
SELENIUM EXCESS
COLD
LACK OF SHELTER
PARASITISM
GROWTH
HIGH MILK YIELD
ACETONAEMIA
INFECTIOUS DISEASES
TICKS
HOOF TROUBLES
STRESS
HYPOTHALAMUS
ANTERIOR-PITUITORY
FSH LH LACK
DELAYED OVULATION
OVULATION FAILURE
ANOVULATION
REGRESSION ATRESIA
CYSTIC OVARIES
FOLLICULAR CYST
LUTEAL CYST

1.3.3. Persistent Corpus luteum: There is little firm evidence that persistence of corpus luteum can occur in absence of uterine lesions. In our opinion that most of the cases of persistent corpus luteum in absence of uterine lesions are incorrectly diagnosed and are due to silent heat or non detected oestrus. The only sure way of reaching a true diagnosis would be sequential rectal palpation or the use of repeat milk or blood progesterone determinations. Gross uterine inflammation or pyometra can be indentified on rectal palpation. The condition can be treated by prostaglandin $F_2\alpha$ followed by antibiotics.

Ovulatory defects are due to two causes : endocrine deficiency or inbalance and mechanical factors. If the quantity of anterior pituitory hormone released is insufficient or its timing is incorrect (This is particularly true of LH) then ovulation is delayed or fails to occur. In small number of cases because of extensive adhesions of ovarian bursa to the surface of ovary the physical process of ovulation is prevented.

1.3.4 Cystic degeneration of ovaries : Ovaries are said to be cystic when they contain one or more persistent fluid filled structures larger than mature follicle i.e. more than 2 to 3 cm in diameter. Cysts arise on account of anovulation. Instead of follicles getting attretic continue to increase in size and persist consequently it alters normal cyclical activity of ovary so that the cow becomes nymphomonic.

David et al (1971) reported 4.2% incidence of cystic ovarian disease in UK. The relationship between yield and incidence of cystic ovaries has been shown by Hendrikson (1956). A feeding of high protein diet appears to be predisposing factor. It is possible that

the disease may be associated with the stress of production and husbandry.

ß carotene deficiency which results in impaired oestradiol 17-ß secretion by ovary has also been shown to predispose to cystic disease perhaps because of the failure to stimulate the ovulatory LH surge (Lothammer et al 1978).

Traditionally ovarian cysts have been classified as either follicular cysts which are thin walled, frequently multiple and with little or no luteal tissue in the cyst wall while luteal or luteinised cysts are thick walled more usually single and with large quantity of luteal tissue present in the cyst wall.

Cows with follicular type of cysts are nymphomonic with excessive prolonged signs of oestrus and shortened intervals between heats. The mucus discharge is watery, easily breaks. The luteal cyst usually results in a cessation of cyclical activity. The cyst functions as persistent corpus luteum. If the luteal cysts are left untreated then a large proportion of the cows become virilised (Arthur, 1959). These individuals will develop masculine conformation and behave like bulls.

Spontaneous recovery may occur to the extent of 5.0% in cows that develop cysts within 45 days of calving (Morrow, 1966). Manual rupture on rectal palpation can be done but it can cause haemorrhage and bursal adhesions if one is not careful. Follicular cysts can be treated by HCG (LH) and GnRH. A dose of 3000 to 4500 IU of HCG is recommended. Elmore et al (1975) treated cows having cystic ovaries with GnRH - HCG combined therapy, resulted with 55%

conception rate to first service. Amongst the cows he treated majority had luteal cysts.

If cystic ovaries are left untreated there is development of mucometra in which there is distension of uterus with mucoid fluid and thinning of uterine wall. An incidence of 7 to 8% of cystic ovaries has been reported in indigenous cattle of milch breed Tharparkar, Sahiwal, and Gir (Bhosrekar, 1973).

1.3.5. Delayed Ovulations : An incidence of 18% delayed ovulation has been reported in South Africa (Van Rensburg and Devos 1962). The delay was less than 48 hrs. in 85% of cases while more than 48 hrs. in 15% cases. A much lower incidence of 2% in "repeat breeder" cows has been reported by Zemjanis (1980). Several authors have noted that certain cows have an apparent prolonged follicular phase of oestrus cycle as determined by low progesterone in blood and milk (Erb et al 1976; Bulman and Lamming, 1978; and Jackson et al 1979).

Delayed ovulations is generally assumed to be one of the causes of infertility in cows that are referred to as cyclical non breeders. These cases occur because of incorrect timing of LH surge or surge of LH getting delayed. Such cases could be treated by intra muscular injection of GnRH at the time of service or AI in which case ovulation usually occurs within 24 hours.

1.3.6 Anovulations : Follicles become attretic and may predispose for anoestrus. Diagnosis can be done by rectal palpation. In cases of anovulatory heats ovaries are rounded ones. Treatment by administration of GnRH can be done on 13th day of the cycle and doing AI on observed heat with good results (Bhosrekar et al 1986).

1.4. Reproductive diseases : The diseases like Brucellosis, Campylobacteriosis are dreadful diseases causing respectively abortions at 5 to 7 months of pregnancy and embryonic mortality. In case of campylobacteriosis cows repeat at irregular intervals. These diseases are self limiting and found only in breedable mature animals and therefore, more care should be exercised while introducing new animal into the herd. While purchasing animals, testing has to be carried out and regular quarantine has to be exercised. Natural service by bulls should be strictly avoided and to avoid the prevalence of the diseases 100% artificial insemination practice by semen from clean bulls should be adopted. Similarly Trichomoniasis (a protozoan disease) is also transmitted through natural service causing early abortions. The protozoa are harboured in cervix and uterus as well as prepuce of bulls. This disease can be prevented by adopting 100% artificial Insemination by the semen from clean bulls. Leptospirosis, listeriosis also cause abortions in the last three months of pregnancy. Abortion may be accompanied by retention of afterbirth and followed by endometritis. Bovine leptospirosis has a wide spread distribution in USA. Diagnosis can be done by agglutination test on maternal blood or preferably by isolation of the organisms from foetal aqueoushumour.

1.4.2 IBR - IPV abortion : The infectious bovine rhinotrachaetis as well as infectious pustular vulvo vaginitis and infectious bovine balonoposthitis has been identified as the group of Herpes Virus. Pregnant animals abort. This causes specific inflammation of the vulvo-vaginal mucosa and of the glans penis and prepuce and can cause a venereal disease which may be spread by coitus. Heifers are more often

severely affected than cows. The vulval lips swell and become tender and mucosa get deeply congested. Small red vesicles develop on the mucosa. The condition is most painful and is marked by restlessness, there may be fever, reduction in appetite and milk yield. IBR - IPV virus is of the chief veterinary importance as a cause of respiratory disease especially in young feed-lot cattle.

The abortions may occur from 3rd week to 3rd month. The autolysis of aborted foetus is seen. After birth is retained. IBR virus can survive in the frozen semen and intrauterine insemination causes endometritis and temporary infertility (Kandrick and MCEntee 1987). IBR virus has the low morbidity though has a wide distribution in cattle which suggests, that the stress of an unfavourable environment or concomitant disease is necessary to give expression to this infection.

In bulls, infection may develop severe degeneration of seminiferous tubules with resultant infertility for 3 to 4 months duration. The IBR-IPV can be diagnosed with the help of Elisa technique.

1.4.3. Specific bovine venereal epididymitis and vaginitis (Epivag) : This virus is also similar to IBR-IPV virus. This disease is specific in cattle and confined to east and central Africa. The disease is transmitted by coitus and is characterised by muco purulent discharge in female and in some cases permanent lesions in the uterine tubes and by hardening and swelling of epididymis in male.

1.4.4. Mycotic abortions : Mycotic abortions due to Aspergillus and Absidia have occured in cattle. Most of the abortions take place in seventh or eighth month of pregnancy. The endometrium may show extensive lesions from which there is very slow recovery and possibly permanent sterility. The disease occurs sporadically and show winter incidence.

Other infectious diseases like Foot and Mouth also cause abortions occasionally.

The cattle herd should be regularly got tested for Brucellosis, Tuberculosis and Johnes disease. They should be regularly vaccinated for Foot and Mouth, Haemorrhagic Septicaemia, Black quarter and Rinderpest diseases.

Any febrile condition in cows may cause infertility therefore, cows should be immediately attended for any febrile diseases, parasitic infestations, Foot rot, overgrown hooves, and injuries etc. Cases of retention of placenta, dystokia and prolapse of vagina/uterus should be attended immediately to avoid further complications like endometritis, metritis, salphingitis, oopheritis, cervicitis and impatent salphinx.

Placenta is normally expelled by cows in 4 to 6 hours after calving and by buffaloes in 6 to 8 hours (Bhosrekar and Sharma, 1972). The placenta or after birth, if gets retained for more than 8 hours, is called retained placenta. Normally it is advised to wait till 12 hours in summer and 24 hours in winter and not to interfere in normal expulsion. If still it is retained then intervention is required. It is author's experience that keeping 2 furea tablets one in each horn will reduce down the infection and inflammation causing expulsion. It is not

advisable to remove placenta manually but the hanging placenta can be cut to 6 inch size so that it will not touch the ground even on sitting of the animal. The tying of stone or shoe to the placenta should be avoided.

Cases of dystokia should be carefully handled in time before it gets delayed as delay may cause death of foetus, emphysema and difficulty in releasing the dystokia. Sterilized instruments, disinfected ropes should be used while relieving the dystokia, lot of vasaline should be used for lubrication. The foetus should be pushed forward and should be corrected first before pulling out. Before putting hands inside the uterus, the nails should be trimmed, hands should be thoroughly washed, disinfected. While pulling foetus care should be taken to avoid injuries to uterine tissue and after relieving dystokia use of abundent antibiotics should be done to avoid further infections.

Caeserian operation can be done and calf can be taken out if diagnosis is done in time.

Prolapse of uterus is again a condition which will cause proliferation of infection in the uterus and subsequent infertility. Prolapse can be pre or post parturient. Continuous loading of the reproductive organs, because of sitting of cows on uneven surface or inclined floors lead to loosening of broad ligaments of uterus and may result in prolapse. Prolapse is very common in buffaloes and old cows. On prolapse the organs should be cleaned, disinfected by using potassium permangnate lotion. If swollen because of delay, adrenaline injection or ice packs can be given, when both things are not at hand sugar paste can be applied, which will help in reducing oedema. The

prolapsed organ can be reduced carefully and truss can be applied to avoid recurrance. Epidural anaesthesia can be given.

Repeat breeding occurs even if some infection is retained, therefore broad spectrum antibiotics and to avoid anaerobic infection to settle use of metranedazole should be given with gentamicin in proper dosage.

Because of inflammatory condition of uterus the involution gets delayed causing delay in post partum occurance of oestrus and longer intercalving period. As a precaution the calving boxes/place should be clean as uterus after calving is wide open and provide a conducive atmosphere for proliferation of infection.

2.0 Role of cow owner/farmer in Breeding Management :

Cow owner has a very important role to play. Housing, feeding and heat detection are the functions he has to carryout. He has to look after general health of the cow and carryout preventive vaccinations against diseases and give timely clinical help on requirement.

2.1.1 Housing : The housing for cow can be a very simple structure, which should be comfortable to the animal. It should be able to protect the animal from direct sun, rain and chilling weather. The house should have "Pucca" floor so that wash water and urine should get drained out. The floor should be antislippery as wet floor can cause slipping of animals causing fractures, wounds. Cows should be housed preferably in loose housing having a floor space of 150 sq.ft. per head. Abundent cool drinking water

should be made available to cows. In loose housing the cows get sufficient exercise which help in proper digestion at the same time it helps in heat detection. Cows also remain comparatively clean. The cow barn should be airy. The roof should be at least 12' high. The feed mangers should be wide and deep at the 2 to 2 1/2' height from ground. The mangers should be protected so that cows will not trample feed and fodder and spoil it.

2.1.2 Feeding : In recent years studies on the effect of nutrition on reproduction in cattle have increased. The nutritional deficiencies or excesses cause infertility. They may act via the hypothalamus and anterior pituitory, thus influencing the production of gonadotrophins or directly on the ovaries thus influencing oogenesis and the endocrine function. Perhaps nutrition influences sperm transport, fertilization, early cell division and the development of embryo and foetus.

Nutrition affects the age at puberty. As the feeding level increases, age at puberty decreases (Witttbank et al 1966). Immediately after puberty the heifer is under considerable stress since she is growing continuously to physical maturity while conceiving and maintaining pregnancy to full term.

The most severe effect of inadequate feeding is the cessation of cyclical activity. Although other less severe manifestations are silent heat, ovulatory defects, conception failure and fetal and embryonic death.

Although protein, vitamin and mineral deficiencies are capable of producing poor fertility, the main effect

is that of deficient energy intake. This has been demonstrated in a study by Leaver (1977).

Normally our farmers give last preferance in feeding to cows. The left over from buffaloes and bullocks is fed to cows. The cows remain unthrifty, emaciated. Under these circumstances they remain anoestrus. They show heat only when rains make the green grass available to them for grazing.

Under feeding of energy affects growth, delays maturity causes under development of reproductive organs, lowered conception rate and repeat breeding. Under feeding of protein reduces conception rate increases services per conception on the contrary high levels of protein in the diet of dairy cows increases incidence of anoestrus and predispose them to cystic ovaries and high levels of energy to fatty cow syndrome.

2.1.3 Specific nutrients : Major minerals are available to cow through grazing grasses and therefore deficiency is unlikely to occur for major minerals.

Trace elements like, copper, cobalt, mangnese, Iodine, selenium are very important from fertility point of view.

Usually poor fertility will be seen when copper and cobalt deficiency is present. Mangnese deficiency has been shown to have adverse effect on fertility with anoestrus, poor conception rates and the birth of deformed calves. (Hignett, 1956; Rojas et al, 1965) Iodine deficiency below 0.8 PPM (Alderman, 1970) can cause infertility such as abortion, still birth, weak calves, fertilization failure and embryonic death.

Selenium deficiency causes placental retention. It is associated with Vit. E.

Vitamin A : Vitamin A deficiency causes delay in onset of puberty in heifers, deficiency of ß carotene results largely in delayed ovulation cases, silent heat and anovulation with follicular cyst formation.

It is necessary to calculate the requirements of cows for maintenance and production and then obtain accurate information about the precise quantities to be fed. Mineral licks or mineral mixture containing major as well as trace elements should be given.

Faulty feeding : Over feeding of energy or fatty diets causes animals to develop fatty tendancy. The fat mainly gets deposited in bursa of ovary causing fertility failures.

Feeding of subterranian clover, Red clover, Berseem and lucerne to cows/heifers as a sole roughage causes ovarian cysts, vulval and cervical enlargements and poor conception rates because of presence of phytosterols in these legumes. Similarly feeding of sugar cane tops, paddy straws, beet roots or kale during winter as a sole roughage causes loss of uterine tone, watery mucus, silent oestrus and poor conception rate because these roughages contain oxalates which bind calcium and will not make it available for the animals. Feeding these fodders along with other roughages like, sorghum, maize, oats or Barley can improve the situation to great extent.

The indigenous cows serve as seed bed for production of high yielding cross-bred cows and hence they

should be looked after at par with other animals and should be given balanced feeding.

Age and body size : Age and body size at first breeding in heifers is greatly influenced by management practices that affect growth and development of animal. In indigenous cattle the age at first breeding and conception was found to be 26.0, 23.9, and 25.1 months and 28.7, 26.0 and 26 months respectively in Tharparkar, Sahiwal and Red sindhi. While in cross bred heifers it was 18 months and 20.2 months respectively. This shows by cross-breeding the efficiency of conversion is enhanced and desired body size is attained at an early age (Bhosrekar, 1976). If the crossbred heifers are bred, earlier than 18 months conception rate is poor. Age as well as body weight both are important from physiological as well as life time production point of view. Heifers of local indigenous breed and buffaloes breed at higher age.

Post partum breeding : The physiological anoestrus is present till involution of uterus is complete. It takes 23 to 30 days in exotic crosses. It is much earlier in drought breeds. In buffaloes and indigenous milk breeds complete involution requires upto 40 days. Therefore, at first heat appearing after 40 days, cows should be inseminated. Cows not showing oestrus till 90 days post partum should be examined. The conception rate is better between 60 to 120 days. After 120 days post partum the fertility gets reduced. Body weight, after calving, determines the appearance of oestrus as well as conception. The loss of body weight on account of calving should be regained at the earliest.

2.2 Heat detection : Another important responsibility of cow owner is heat detection. He should know the heat symptoms and should be able to detect the behavioural changes in cows. For that he should visit the cow barn morning and evening, observe the cows carefully, see if there is any mucus disharge, if she is restless, off feed mounting etc. About 80 to 85% cases can be detected correctly in heat by herdman. Other, methods of heat detection are available like heat mount detector 'KAMAR', using tail paste or paint, androgenised cows, trained dogs and teaser bulls as well as measuring mucus resistance by a electronic probe 'ESTRON'.

In range cattle heat mounts as well as teaser bull can be used in combination. Buffaloes can be detected in heat from typical sympotms like swelling of vulva, frequent micturition and reddening of nictating membrane.

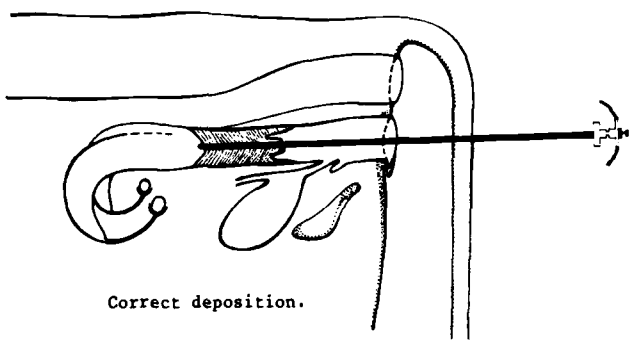
Majority of the cows go infertile because of the failure in heat detection and timely insemination not being done. Heat detection is crucks of the problem. On heat detection, reporting to inseminator and getting the cow/buffalo inseminated in time is also equally important. From the mucus discharge optimum time for AI can be worked out. In good standing heat in healthy cow the mucus discharge is roapy, copious and transparent like child's saliva. In early heat the mucus discharge is watery, breaking, while in late heat the mucus discharge is scanty and thick. Cows should be got inseminated in mid-standing oestrus when above type of mucus discharge is seen, about 60 to 65% conception rate is expected.

3.0 Role of Inseminator :

The inseminator should be a perfectly trained man. He should be able to handle frozen semen, should be able to detect proper stage of oestrus, should be able to deposit semen at proper site of deposition and should be able to use proper technique of thawing of frozen semen, mounting of A.I. gun and introduce hygienically at proper site and deposit semen.

Holding of cervix, keeping organs in horizontal plane and the body of uterus not to be caught should be known to the inseminator (See Fig. 26) The semen should be deposited in the body of the uterus 1/2 a cm ahead of internal opening of cervix to get optimal results.

Fig. 26



Correct deposition.

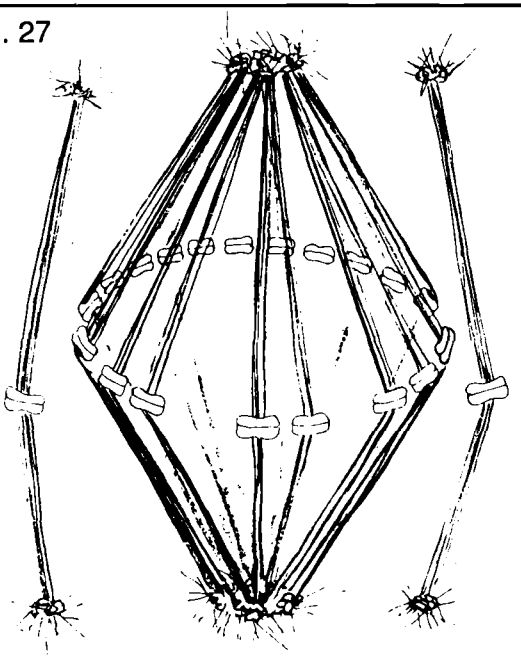
The cows should be inseminated in proper time and AI should not be too delayed as the ovum has little life span as compared to sperm and sperm also require some time before they could fertilize ovum. According to Hunter (1985), the artificial insemination industry is expanding on a worldwide scale and indeed more

and more farmers are using "do it yourself" procedures for insemination. In this situation it seems especially important to explain why delayed insemination has a deleterious influence on conception rate in cattle and must be avoided. The single egg is more susceptible to the influence of aging in the female tract than the millions of spermatozoa introduced at insemination and this is expressed in a malfunction of egg organelles such as cortical granules and the second meotic spindle. Delayed insemination, therefore, lends to abnormal fertilization (or complete failure of fertilization) followed by early death of egg or embryo since cow eggs are thought to remain fully viable for only 8 to 10 hours after ovulatin. Preovulatory insemination is essential for establishment of a population of competent spermatozoa in the oviducts close to the site of fertilization. Frozen-thawed semen is probably best inseminated 12-18 hours before ovulation. The stage of oestrus should be properly detected for this purpose. Insemination too soon (i.e. premature insemination) puts the spermatozoa at risk from aging in female tract, where as insemination too late compromises the ovulated egg.

First the nuclear elements in the newly ovulated egg consists of metaphase arrangements of chromosomes on the second meotic spindle. These await activation by the fertilizing spermatozoan. In absence of such activation and after only a small number of hours the microtubules of the meotic spindle commence to become disorganised with pairs of microtubules gradually escaping laterally from the spindle apparatus to wander in the vittelus (Szollosi, 1975). The actual loss of chromosomes from the metaphase plate is a concomitant process (Fig. 27). Thus even if the egg should subsequently be penetrated by a

spermatozoan, normal fertilization and formation of zygote with correct deploid complement of chromosomes can not endue. Early embryonic death is the most frequent sequel.

Fig. 27



Second orgenelles concerned with the defence mechanism against fertilization by more than one sperm, the so called block to polyspermy consist of vast number of minute vesicles distributed around the egg surface just beneath the plasma membrane (Szollosi, 1962, 1967; Flechon, 1970).

Each vesicle measures less than 1 m μ in diameter and is rather misleading referred to as corticle granules (Fig. 28) At the time of activation by the spermatozoan the vesicles fuse with overlying plasma membrane to release their contents in to the perivetteline space

(Fig. 28).This step leads to alteration of zona pelucida conferring the block to polyspermy. The essential point of the cortical granules is that they migrate from golgi regions with in egg to take up their position below the plasma membrane just before ovulation (Szollosi, 1967). Here they remain for small number of hours until as post ovulatory aging sets in. They begin to swell and wander away from the egg's surface deeper into the cortex. In this despersed condition release of contents of the cortical granules in to the perivitteline space can not follow activation of the egg so the block to Polyspermy is not instigated and the egg remains to risk from multiple fertilization.

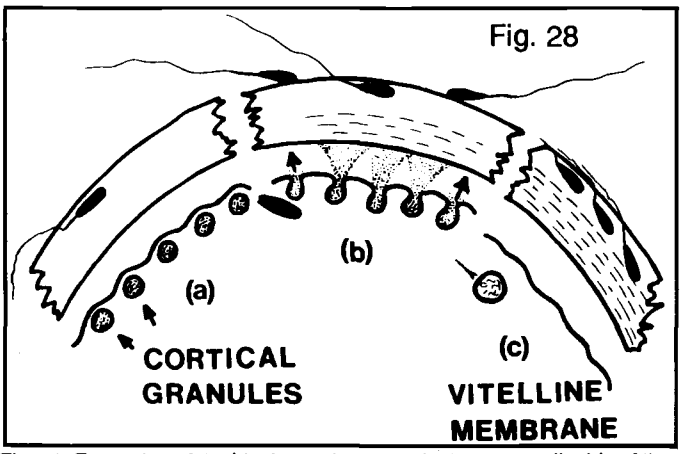


Fig. 28. Formation of the block to polyspermy in the zona pellucida of the mammalian egg.

(a) Fertilising spermatozoon has penetrated the zona, but the cortical granules remain intact just beneath the vitelline (plasma) membrane.

(b) The fertilising spermatozoon has now entered the egg cytoplasm triggering an 'activation' of the cortical granules. Their contents are released by a process of membrane fusion, and bring about enzymatic changes in the inner portion of the zona pellucida - the block to polyspermy.

(c) The head of the fertilising spermatozoon has evolved into a male pronucleus. Supplementary spermatozoa can still penetrate the outermost portion of the zona, but are unable to traverse this membrane to enter the egg cytoplasm; the latter has contracted slightly in response to fertilisation.

An additional feature of the post ovulatory situation is that regulation of the number of spermatozoa passing from the uterus to the site of fertilization in the mid region of the oviduct is considerably less effective. This is because of the musculature of the oviduct isthmus is more relaxed and oedema of the mucosa is reduced (Hunter 1977), Hence, in contrast to the general notion of poorer sperm transport with delayed insemination, the oppoite may frequently be the case when the barrier presented by the cervix is overcome by the insemination straw, Accordingly, the risk of polyspermy is further increased since the chances of the aging egg being confronted simultaneously by two or more competent spermatozoa are increased.

Degenerative changes in the egg and post ovulatory changes in the condition of the tract together will lead to a reduced incidence of normal fertilization and the enhanced incidance of embryonic death as a overall result of late insemination.

4. Semen :

The chilled liquid semen or frozen semen should be from the bulls which have disease free status. In case of chilled liquid semen the care should be taken to avoid contamination in handling. It should be utilized before 48 hours to expect optimal results. The agitational shock should be avoided and cold chain should be maintained till utilization.

In case of frozen semen, the frozen semen should be always kept under liquid nitrogen. The straw should not get exposed to critical temperature. Straw once taken out of the container should not be put back in liquid nitrogen as the sperm can not bear double reversal shock and will get damaged.

The frozen semen should come from highly pedigreed merited bulls free from transmitting diseases like Brucellosis, Campylobacteriosis, Trichomoniasis, Leptospirosis, Listeriosis, IBR-IPV, Epivag, Foot and Mouth etc.

The bulls should be regularly tested for Tuberculosis and Johne's disease and positive reactors should be culled.

Frozen semen used should be of high standard having minimum of 10 to 12 million live motile sperm at the time of insemination after thawing. That means at packaging each straw/dose should contain 30 millions spermatozoa with initial 80% motility. The post thaw motility should not be less than 50% in any case.

The frozen semen should be free from pathogenic organisms and even nonpathogenic should not be more than 750 per ml. The frozen semen of this standard should be from fertile bulls. At BAIF's Semen Freezing Laboratory these standards are maintained strictly.

If these standards are fulfilled about 60 to 65% conception rate is expected at first AI if all other conditions are optimal.

Any thing going wrong in above four aspects of breeding management the results are likely to be affected.

4.2 Insemination Conditions : The AI should not be carried out in sun and should be carried out in shed. The AI should be carried out in cooler hours of the

day. Afternoon has high ambient temperature so also high body temperature of cow which affects uterine environment. It has been observed that cooler hours are more conducive for AI work and for getting higher conception rate.

4.3 Thawing of frozen semen : Thawing should also be carried out in warm water at 35° C for 30 seconds. Lower fertility is reported if thawed only for 12 seconds at 35° C. Hand thawing, pocket thawing should not be used as it will not provide uniformity in thawing condition. Only one straw should be thawed at a time.

4.4 Psychological disturbances : Possibility of psychological disturbances resulting in lowered fertility can not be ruled out. Mistreatment of cows inheat may lower the fertility. The pathways involved in lowered fertility from mistreatment seem to involve mainly the hormone of the adrenal medulla, epinephrine and the hormone of the pituitory, ACTH which causes the adrenal cortex to rlease the adrenocortical hormones.

Salisbury and Van Demark (1962) reported higher conception rates of 72.4 and 62.7 percent in quiet and slightly nervous cows as against 60% in highly nervous cows.

The conditions, therefore, should be quiet, fearless and less disturbing at the time of artificial insemination for getting optimum fertility.

4.5. Possible Immunological causes : Antibodies built up against sperm experimentally, either in different species or in the same species were capable of preventing fertilization when brought in direct contact

with the sperm in sufficient concentration. Kiddy et al (1958) found interference with fertilization by antibodies in rabbits. Similar results were also obtained by Menge et al (1960) in cattle. It is believed that antibodies against sperm are built up in the genital tract of repeat breeder cow getting repeated insemination from the semen of one bull. These antibodies cause clumping of spermatozoa causing intereference in fertilization. Even if the fertilization is successful there will be an early embryonic mortality.

Changing of bull or heterospermic insemination solves the problem.

Similarly allergy is developed against eggyolk or milk protein, (eggyolk or milk is used usually in the diluent for sperm), which also interferes with fertilization. Cows repeating frequently without apparent cause may suffer from one of such problems. Sexual rest and missing 2 to 3 heats will bring back normal conditions and cows settle on A. I.

Other factors :

Other factors like herd size, season, age, milk yield and suckling also cause lowered conception. Hewett (1968) from Sweden reported significant role of these factors in determining conception rate. According to him management in big herd is poor and therefore heat detection is poor. In small herds the management is better, heat detection is efficient and thus the conception rate is higher. Age of the cow also plays a very significantly important role in getting optimal conception rate. Heifers and old cows show poor conception rate as compared to young cows between 1st to 3rd lactation. Bhosrekar (1973) reported similar

observation. In winter months less green is available, animals are stall fed all through with high energy-low protein rations causing lowered conception and higher repeat breeding as compared to other seasons in Sweden (Hewett, 1968). High yielding cows as well as suckling cows show lower conception rate may be because of higher drain of pituitory LTH hormone for milk production causing less responsiveness to gonadotrophin secretion.

Air Insufflation and Hydrotubation

This technique can be used for diagnosis and treatment of tubal impatency in cows and buffaloes.

Tubal impatency is one of the causes of 'repeat breeding' in cows and buffaloes. Fallopian tube is an important vital link between the ovary and the uterus. Fertilization takes place in isthmus-ampula region of fallopian tube. Roberts (1971) stated that the fallopian tube disorders and its diseases probably occured more commonly than generally assumed and diagnosed.

The tubal impatency is caused as sequel to chronic inflammations of uterus as most of the tubal blocks are reported to be at the utero-tubal junction. Buffaloes are more prone to this affection because of their habits of wallowing in stagnant and dirty water tanks in the country side.

Diagnosis and treatment of the tubal impatency can be done by using uterotubal insufflation and hydrotubation technique. For air insufflation an apparatus described by Kavani and Kodagali (1986)

can be used. This apparatus was originally designed by Chenne Gowda and Abdulla Khan (1975). This consists of :-

1. A dial mannometer calibrated to read between 0 to 300 mm Hg pressure.
2. Rubber tubing (Latex 4 mm. diameter) for assembling different parts.
3. The '3' way 'Y' steel cannula.
4. A 50 cm long stainless steel uterine catheter of 5 mm diameter for cows and 3 mm diameter for heifers. With an opening at the uterine end.
5. A 22 FR Neo Foley's latex balloon catheter having universal aperture for inflating or deflating the rubber balloon near the tip.
6. A rubber blower with one way air valve at the tip and a stopcock to release the air.

Procedure for tubal patency test :

The Neo Foley's latex balloon catheter is sleeved over the metal catheter and the equipment was assembled. The uterine canula is guided in to the cervical canal and the balloon is located at the mid cervix. About 3 to 5 ml of water is injected through the universal aperture to inflate the rubber balloon sufficiently to cause, complete obturation of the cervical canal between cervical annular folds. Air was blown into the uterus using the air blower at a rate of 10 mm Hg pressure per minute. The rate is then reduced to 5 mm Hg pressure per minute. Gradual building up of pressure as recorded by the mannometer is taken as an indication for complete cervical obturation.

When sufficient pressure is built up in the uterine cavity air started escaping through one or both the

fallopian tubes this can be felt by the operators hand. If the pressure falls rapidly it can be taken as indicative of tubal patency. If the rate of fall of the intra uterine pressure is very slow then it can be considered as unilateral tubal impatency. On the otherhand if the intra uterine pressure remains steady, it indicates bilateral tubal impatency.

Treatment : After testing the animal by air insufflation method the antibiotic solution in sterile 5% dextrose or normal saline can be infused under pressure ranging from 200 to 300 mm Hg. The distention of uterus can be felt per rectally. By this technique temporary tubal blocks get dislodged and tubes become patent.

Athman et al (1984) reported that 2.86% and 14.28% cows had unilateral and bilateral impatency of tubes and by using hydrotubation technique 69.57% cows concieved. Kavani and Kodgali (1986) used this technique of hydrotubation in 72 repeat breeder buffaloes having tubal impatency as diagnosed by air insufflation method and reported 55.55%, and 41.66% pregnancy rate in unilateral and bilateral impatency cases.

PSP Test : Diagnosis of impatency of tubes can be done by PSP test in bovines. Typical colour reaction of urine is observed at 30 minutes post infusion. This test is based on the principle that 20 ml of 0.1% phenol-sulphna phtheline (phenolred) infused intrauterinely would be selectively absorbed by the peritonium on escape through patent fellopian tubes and would get excreted through kidneys imparting pinkish or reddish coloration to urine on alkalinisation. No change in colour would be observed in bilateral impatency of tubes.

20 ml 4% Indigo carmine can be also infused in the uterus. In this case bluish green coloration of urine is observed 2 to 2.5 hours following intrauterine infusion of the dye.

Lugal's Iodine for induction of heat :

Preparation of Lugal's Iodine

The lugal's iodine solution can be prepared as follows

Iodum crystals	5 g.
Potassium Iodide	10 g.
Distilled water	100 ml.

From this stock solution 1 ml is taken and mixed with 10 ml warm distilled water and this 20 ml. of lugal's Iodine is infused in the uterus with the help of insemination pipette. This also can be painted on external 'OS' uterus and adjacent area. This treatment is advised for anoestrus. The infusion or painting will cause counterrritant action thereby resulting in hyperaemia of uterine tract. This will help in inducing the cylicity of the cow or buffalo.

Infusion of lugal's Iodine will act as a chemical curator and help replacing the mucus membrane with new tissue, secretion of $PGF_2\alpha$ and causing regression or lysis of corpusluteum and initiating the oestrus cycle.

It also gets absorbed through mucus membrane and helps increase thyroxine production, thereby gearing up the general metabolism. So lugal's Iodine has not only topical but also systemic effect.

Lugal's Iodine should not be too strong. It should not be more than 1% and have a coffee colour when prepared.

CHAPTER XVI

SEMEN PRESERVATION AND ARTIFICIAL INSEMINATION IN GOATS

Recently there has been realization that goat has many facets that makes it suitable for rearing in situations unsuitable for other domestic animals. There is an increasing awarness that goats are playing and can play an important role in meeting the requirements of food for the impoverished and ever rising population.

The goats can provide milk, meat, skin and hair as well as rich manure for the soil. There are recognised breeds of goats which provide milk (e.g. Jamnapari, Beetal, Barberi and Osmanabadi) meat (e.g. Black, Bengal, Malabari, Marwari, Mehsana and Zalwadi goats). Hair (Pashmina goats found in Gilgit, Ladakh also seen in Tehri and Almora districts of U.P.).

As far Maharashtra is concerned there are more of non descript types of goats numbering to 7.56 millions. 1.5 million rural families maintain these and are in business of goat production. About 10 million litres of milk, 26,590 tonnes of meat is produced every year from the goats in Maharashtra. There has been an increasing demand for goat meat in the country as well as abroad and can form a source of earning foreign exchange for the nation.

The goat breeding is required to be put on sound footing by adopting scientific methods of goat production. Artificial Insemination is one of the biotechnology which can help in development of goats.

This technology can be adopted to improve native non descript goats for improving the productive traits like milk or meat.

The artificial insemination in goats is not popular in the country mainly because of the lack of extension, communication and delivery services. If the goat owners are made aware of advantages of artificial insemination this technology can very well be utilized for the development of the goat industry.

Exotic breeds like, Sannen, Alpine can be used for producing crosses for improvement in milk production. Anglo Nubian, Angora could be used for producing cross-breds for improvement in meat production, higher dressing percentage etc.

Bucks are handled basically the same way as bulls for semen collection. Artificial vagina 20 cm long having a diameter of 5.5 cm can be used with rubber linings, cone and collection glass tube for collecting semen. The buck is allowed to mount on an another buck with a semen collector manually diverting the buck's penis into the artificial vagina.

Bucks do not pose any problem for mounting on another buck and for donating semen in artificial vagina. The volume of the semen per ejaculate ranges between 0.2 to 1.2 ml depending upon the health, age and body weight of the buck.

The sperm concentration ranges between 3000 to 4000 x 10^6 per ml. The initial forward motility is more than 80% in healthy adult bucks.

The semen can be utilised after dilution for Artificial Insemination as chilled liquid semen or also can be deep frozen and frozen thawed semen can be utilized.

Trials have been conducted to process buck's semen by diluting it with Tris eggyolk-citric acid-fructose glycerol diluent.

After serveral trials the procedure has been now standardised. Buck's semen can not tolerate more than 5% glycerol, 15% eggyolk and 5 hours equilibration period. So for successful freezing of bucks semen diluent should have 15% eggyolk, 5% glycerol instead of 20% eggyolk and 7% glycerol as in the case for bulls semen.

Optimum equilibration required is 5 hours. The buck's semen can be frozen without washing of spermatozoa successfully. There has been some apprehention that buck's semen can not be preserved without sperm washing as the semen plasma of buck's semen has an enzyme (phospholipase) which coagulates eggyolk but it has been found in our experiments that buck's semen can be diluted and preserved successfully without sperm washing. Sperm washing was done by tris buffer after centrifuging at 3000 RPM for 10 minutes without much advantage.

The diluted semen is packaged in minitube by automatic filling and sealing machine (MT 65) and the straws thus filled and sealed are kept for equilibration of 5 hours. It has been found in our experiments that buck's semen needs slow glycerolisation at 5^0 C for getting optimum results.

It is often very difficult to introduce more than 0.2 ml. of semen in to the cervix of doe. So dilution to a final concentration of 600 million to 120 million live sperm per ml. has been recommended. For successful A.I. a dose of 120 millions of sperm is recommended. Therefore, 3 to 4 straws of semen containing 30 millions each are required per A.I. in goats. The oestrus period in does remains for 36 hours but it varies between 24 to 48 hours. The goat is spontaneous ovulator with the ovulation occuring shortly before the end of oestrus. Therefore, the artificial insemination should always precede the time of ovulation because the survival rate of the egg in reproductive tract is shorter (15-24 hours) than that of sperm (30 to 48 hours). In addition there is time required for sperm capacitation in the reproductive tract of the doe before egg fertilization is possible.

The estrus cycle in does is on an average of 21 days with variation of 18 to 24 days. The breed and eco-climatic effects also influence the cycle length.

Goat is seasonal breeder and the season starts from mid September to the end of December. Goat is seasonally polyestrus that means in one season it shows several heats if not conceived. In our country goats show two seasons of breeding. September to December and February to May. Tropical goats do breed all round the year. Environmental factors play a role in expression of oestrus and its onset. Photoperiodicity i.e. ratio of day light to darkness has been shown to influence the onset of oestrus. Reduction in number of day light hours and decrease in ambient temperature seems to be having effect on promoting oestrus.

It is important to become familiar with the signs of oestrus exhibited by the does. These signs are valuable pieces of information which can be used to determine optimum time for artificial insemination. Following are the symptoms of oestrus.

Increased frequency of bleating, frequent urination and restlessness followed by tail flagging, mounting and intermittent discharge of vaginal mucus. The colour of the mucus membrane of valva changes from pale to bright pink. Valva gets swollen and the doe stands for mounting.

Synchronisation of oestrus has been studied extensively in Western Countries. In order to avoid seasonality, and to have uniform year round milk production oestrus synchronisation has been successfully tried in does.

Ist method involves the use of 21 day progestogen treatment in the form of vaginal sponge. Insertion of sponge is followed by a injection of PMSG (Pregnant Mare Serum Gonadotrophin) 400 IU at the time of removal of sponge. This treatment induces a very high level of oestrus onset with 100% treated does ovulating. The progestogen commonly used is Flourgesterone acetate at applied dose of 45 mg. second method of oestrus control is by means of intra muscular injection of prostaglandin F2 α in the natural form or its synthetic analogue. This is only effective in natural breeding season when does are cycling and when there is a functional corpus luteum. This hormone causes lysis of corpus luteum and triggers the onset of heat. As all the does may not be in the same stage of cycle there is need to give the PFG2 α twice with the interval of 10 to 11 day so that all the does are

covered (Those in early as well as late in the cycle). The dose per 100 kg body weight is 1.25 mg for lutalase a natural PGF while it is 100 to 125 ug for synthetic analogue of PGF.

The Artificial Insemination can be done within 72 to 96 hours after the 2nd injection of PGF2α.

The 3rd method is by controlling the light exposure schedule. It has been known that oestrus cycle and behaviour of goats are modulated by the length of the day light. Dairy goats start their breeding season some 8 to 10 weeks after the longest day of the year. As the days shorten the light sensitive endocrine system of the doe and the buck indentifies the change and the mechanisms are set in motion that brings about the breeding season in the fall.

Taking the advantage of this natural phenomenon the light exposure schedules should be monitored in such a way that breeding cycle sets in and does come in oestrus.

The gestation length in goats is 150 days. Pregnancy diagnosis can be done by ballotment technique or by ultrasound or radiography prognosticators.

Usually kidding percentage ranges from 33 to 69% twinning, 11 to 25% triplets and rarely quadriplets. The sex ratio is 50:50 between male to female kids.

BIBLIOGRAPHY

Abdel Rouf (1960). Acta Endocri. Copenh. Suppl. 49.

Abdou, M.S. and E.L. Guindi, M.M. (1977). Zentral blatt fur veteri. Medizin 24(7) 575-599, (Anim.Breed. Abstr. 44(4) : 1766), (1978).

Abhi, H.L. (1982). Indian J. Anim. Sci. 52(9) 809-811.

Abhi, H.L., Nagpaul, M.P., Sharma T.C. and Grewal A.S. (1973). J. Dairy Sci. 26(2) 107-113.

Adler, H.C. and Rasbech, N.O. (1956). Nord. Vet. Med. 8, 497.

Adler, H.C. (1961). 3rd International Congress. Anim. Reprod. Al. 4; 947.

Adler, H.C., Anderson J.B. and Meding, J.H. (1968). Arsbersten. Inst. Sterilitets Forsk Kevet og Landbohojsk. 9.

Ahmed, K. (1984). Indian J. Dairy Sci. 67(7) 1535-1538.

Aire, T.A. and AKPodje, J.O.(1975). British Vet. J. 131; 146. 51.

Alderman, G (1970) Vet. Rec. 70; 35

Ali, S.B.A., Al Hakim, M.K. and Singh, B.P. (1981). Indian J. Anim. Hlth. 20(1) 9-13.

Allen, C.H. and Almquist, J.O. (1981). J. Anim. Sci. 53(6), 1432-1459.

Almquist, J.O. (1959). A.I. Digest 7(8); 11.

Almquist, J.O. (1975). J. Dairy Sci. 58(3); 420.

Almquist, J.O. (1951). J. Dairy Sci. 34; 819.

Almquist, J.O. and Amann, R.P. (1976). J. Dairy Sci. 59(5) 986-991.

Almquist, J.O., Branas, R.J., Barber, K.A. (1976). J. Anim. Sci. 42(3) 670-76.

Almquist, J.O. and Hale, E.B. (1956). Proc. 3rd International Congr. Anim. Reprod. (Camb) Plannery papers pp 50-59.

Almquist, J.O. and Wiggin, H.B. (1973). A.I. Digest. 21(7); 12.

Almquist, J.O., Hale, E.B. and Amann, r.P. (1968). J. Dairy Sci. 41; 733.

Almquist, J.O. and Zaugg, N.L. (1974). J. Dairy Sci. 57(10), 1211-13.

Altmen, M. (1936). Anim. Kingd. 66; 181.

Altman, P.L. and Dittmer, D.S.(1962). Growth including Reproduction and Morphological Development:Biological Handbook. Federation of Amer. Soc. Exptl. Biol. Washington DC.P. 188.

Anderson, J. (1941). Vet. Rec. 543; 197.

Anderson, J. (1951). Vet. Rec. 63, 733.

Anderson, H.W. and Seath, D.M. (1948). J. Dairy Sci. 31:551-666.

Anderson, J. (1945). Tech. Comm. Bur. Anim. Breed. (Genet.Edinb.) 6, 151.

Anderson, J. (1946). J. Agri. Sci. 36; 258.

Anderson, H., Koefoed, Johnson, H.H. 91965). Arsbersten. Inst. Sterilitets Forsk. KaVet. og/ :amdnpjpksl. 121-131.

Antonynk, V.S. and Bezlyerdnikov; L.G. (1983). Zhivotnovodstva 24; 61. Anim. Breed. Abstr. 70 (Pig news & Information).

Arriola, J.O. and Foote, R.H. (1987). J.Dairy Sci. 70; 1664-1670.
Arthur, G.H. (1959). Vet. Rec. 71, 598.
Asdell, S.A. (1946). Patterns of Mammalion Reproduction, Constock, Ithaca Newyork.
Athman, K.V., Neelakanta Iyer, C.P. and Prabhakaran Nair (1984). Indian J. Animal Reproduction 4 (2) 30-33.
Austin, C.R. (1951). Aust. J. Sci. Res. B 4; 581.
Austin, C.R. and Bishop M.W.H. (1958). Proc. Roy. Sco. B 149. 234-241.
Austin, C.R. (1959). In cole, H.H. and Cupps P.T. Reproduction in Domestic Animals Vol. II Academic Press N.Y. & London pp 399.
Ax, R.L. and Lenz R.W. (1987). J. Dairy Sci. 90 (7) 1477-1486.
Baier, W., Leidl. W. and Greiff, W. (1957). Berd. Mouch. Tierasztl. Wschr. 70; 229.
Baker, F.N. and Van Demark, N.L. (1952). J. Dairy Sci. 35; 489.
Baker, F.N., Gragle, R.G., Salisbury, G.W. and Van Demark, N.L. (1957). Fert. Steril. 8, 149.
Bane, A. (1954). Acta. Agric. Scand. 4, 95-208.
Bandopadhyay, S.K. and Roy, D.J. (1976). Indian J. Anim. Hlth. 15 (1) 37-41.
Barbulscu, I. (1967). Anim. Breed. Abstr. 35 (1) : 307.
Barrett, A.J. (1972). Lysozome enzymes in "Lysozomes", A. Laboratory handbook pp 46-135. Ed. J.T. Dingle. North Holland Amsterdam Landon.
Basirov, E.B. (1960). Anim. Breed. Abstr. 28; 1294.
Basu, P.T. and Berry, R.O. (1948). J. Anim. Sci. I, 447.
Beck, G.H. and Salisbury, G.W. (1943). J. Dairy Sci. 26, 487.
Becker, W.C., Senger, P.L., Aalseth, E.P. and Marshell, C.E. (1977). J. Anim. Sci. 44 (6) 1067-1071.
Bedford, J.M. and Chang. H.C. (1962 b). Nature (Lond) 193; 898.
Bedford, J.M. and Chang, M.C. (1962 a). Amer. J. Physiol. 202; 179.
Bedford, J.M. (1972) Amer. J. Anat. 133, 213.
Bedjakov, S.P. (1959). Veterinariya. 36 (1); 78.
Belorkar, P.M., Dhami, A.J. and Kodagali, S.B. (1988). Indian Vet. J. 65 (a) 809-812.
Bennetts, H.W. (1947). Aust. Vet. J. 23; 10.
Bhandari, N., Chavan, R.A.S. and Mathew, A. (1982). Indian J. Anim. Sci. 52 (a) 812-813.
Bhavsar, B.K., Patel, K.S. and Kodagali, S.B. (1986). Indian J. Anim. Reprod. 7; 1.
Bhagoji, G.P. (1985). Ph. D. Thesis Guj. Agric. Univ. Anand.
Bhosrekar, M.R. and Bhatnagar, D.S. (1971). Indian J. Anim. Prod. 2(1) 13-21.
Bhosrekar, M.R. and Sharma K.N.S. (1972). Indian J. Anim. Prod. 3(1) : 8-15.
Bhosrekar, M.R. and Nagpaul, P.K. (1972). Indian J. Dairy Sci. 25 (3) 194-195.

Bhosrekar, M.R. (1973) Indian Vet. j 50(5) 418-429
Bhosrekar, M.R. and Razdan, M.N. (1973). Indian J. Anim. Sci. 43 (3) 196-200.
Bhosrekar, M.R. (1975). Gosamvardhana (Jan.-Feb.) pp 7-9.
Bhosrekar, M.R. and Ganguli, N.C. (1976). J. Reprod. fert. 4,8, 215-216.
Bhosrekar, M.R. (1975). Biochemical Studies on keeping quality of frozen buffalo semen, Ph.D. Thesis NDRI, Karnal, Punjab University, Chandigarh.
Bhosrekar, M.R., Nagpaul, P.K. and Goel, V.C. (1973). Proc. Indian Dairy Industry Conf. Baroda.
Bhosrekar, M.R. and Ganguli, N.C. (1974). In XIX International Dairy Congr. IE New Delhi.
Bhosrekar, M.R. (1976) Tropical Anim. Health Prod. 8;83-84.
Bhosrekar, M.R., Sane, S.N., Sharma, M.S. and Rangnekar, D.V. (1980). Indian J. Anim. Sci. 50 (2) 113-118.
Bhosrekar, M.R. (1980). Indian Vet. J. 57 (10) 806-810.
Bhosrekar, M.R., Char, S.N., and Rangnekar, D.V. (1980). Indian J. Dairy Sci. 33 (1) 125-127.
Bhosrekar, M.R. (1981). Indian Vet. J. 58 (10) 784-789.
Bhosrekar, M.R., Char, S.N., Waghmare, B.S. Thakur, Y.A., Badve, V.C. and Rangnekar, D.V. (1982). Indian J. Anim. Sci. 52 (7), 579-83.
Bhosrekar, M.R. (1983). Indian J. Anim. Repord. 4 (1) 92-93.
Bhosrekar, M.R., Char, S.N. and Rangnekar D.V. (1983). Indian J. Anim. Reprod. 4 (2) 83-84.
Bhosrekar, M.R., Badve, V.C., Char, S.N. and Rangnekar, D.V. (1983). Indian J. Anim. Repord. 4 (2) 94-95.
Bhosrekar, M.R. (1984). Indian J. Anim. Reprod. 5 (1). 119-123.
Bhosrekar, M.R., Char, S.N., Patil, B.R. and Rangnekar, D.V. (1984). Indian J. Anim. Sci. 54 (12) 1126-1130.
Bhosrekar, M.R., Joshi, A.G., Pande, A.B. and Mangurkar, B.R. (1985). Indian J. Anim. Reprod. 6 (2) 82-84.
Bhosrekar, M.R; Inamdar, A.J., Joshi, B.M., Phadnis, Y.P.; Lokhande, S.M. and Mangurkar, B.R. (1986). Indian Vet. J. 63; 833-837.
Bhosrekar, M.R., Nisal, P.R., Purohit, J.R., Pande, A.B. and Joshi A.L. (1986). Indian J. Anim. Sci. 56 (2) 214-218.
Bhosrekar, M.R., Purohit, J.R., Pande, A.B. and Mangurkar, B.R. (1986). Indian J. Dairy Sci. 39; 316.
Bhosrekar M.R., Mangurkar B.R., Patil S.G., Purohit J.R., Humblot, P., and Thibier, M, (1986) Rev. Elev. Med. Vet. Pays. Trop. 39 (1) 129-137.
Bhosrekar, M.R., Joshi, A.L., Sabne, K.S., Nagendra, G; Waghmare, B.S. and Purohit J.R. (1986). Indian J. Dairy Sci. 39 (3) 215-20.
Bhosrekar, M.R., Thole, N.S., Joshi, A.L., Purohit, J.R, Pande, A.B. and Mangurkar, B.R. (1988). Indian J. Anim. Sci. 58 (5) 619-622.
Bhosrekar, M.R., Purohit, J.R., Thole, N.S., Nisal, P.R., Pande, A.B. and Mangurkar, B.R. (1988). Indian J. Anim. Sci. 58 (4) 510-514.

Bhosrekar, M.R. (1988). A.I. News 3 (1); 11-12.
Bhosrekar, M.R. (1988). A.I. News 3 (1); 13.
Bhosrekar, M.R. (1988). A.I. News 3 (2); 12.
Bhosrekar, M.R., Purohit, J.R., Pande, A.B. and Mangurkar, B.R. (1988). Indian J. Anim. Sci. 58 (9) 1073-1074.
Bhosrekar, M.R., Purohit, J.R., Pande, A.B. and Mangurkar, B.R. (1988). Indian J. Anim. Reprod. 9 (2) 109-114.
Bhosrekar, M.R., Purohit, J.R., Pande, A.B. and Mangurkar, B.R. (1989). A.I. News. 4 (1); 9.
Bhosrekar, M.R., Purohit, J.R., Mokashi, S., Mangurkar, B.R. (1989). IDRC Project report.
Bhosrekar, M.R., Purohit, J.R., Mangurkar, B.R. (1989). Paper submitted to All India Conf. Anim. Repord. Anand. 1989.
Bialy, G. and Smith, V.R. (1958). J. Dairy Sci. 41, 422-28.
Bishop, D.W. (1962a). Physiol. Rev. 42 : 1.
Bishop, M.W.H., Campbell, R.C., Hancock, J.L. and Walton, A. (1959). J. Agri. Sci. 44; 227.
Bishop, D.W. and Mathews, M.P. (1952). Science. 115; 209.
Blackshaw, A.W. (1954.) Aust. J. Biol. Sci. 7 ; 573.
Blackshaw, A.W. (1955). Aust. Vet. J. 31, 238.
Blom, E. (1944). Sked. Vet. Tidskr. 35; 779-89. (ABA : 14; 84, 1945)
Blom, E. (1945). Vet. J. 102; 252-259.
Blom, E. (1955). Zootech. E. Vet. 10; 427.
Blom, E. (1959). Nature (Lond) 183; 1280.
Blom, E. (1968). Nord. Vet. Med. 20; 279.
Blom, E. (1966). Nature, 209, 739.
Bodansky J. (1932). J. Biol. Chem. 99; 197.
Bomstein, R.A. and Steberi, E.A. (1957). Exp. Cell. Res. 12, 254-264.
Bonadona, T. (1956). Plennery papers 3rd Internat. Congr. Anim. Reprod. (Camb) 105-112.
Bonadona, T., Caretta, A. and Corrias, A. (1953). Zootech. E. Vet. 8; 369.
Bonfert, A. (1956a). Tier arztl. Wschr. 63(11). (ABA 24; 1096).
Bonfert, A. (1953). Tierarztl. Umsch. 8, 414.
Bower, R.E. (Jr.), Crabo, B.G., Pace, M.M. and Graham, E.F. (1973). J. Anim. Sci. 36(2) 319.
Boyd, E.N., Perkins, J.R., Olds, D. and Seath, D.M. (1954). J. Dairy Sci. 37, 650.
Bratton, R.W., Musgrave, S.D., Dunn, H.O., Foote, R.H. and Henderson, C.R. (1956). J. Anim. Sci. 15; 1296-1297.
Britt, J.H., Morrow, D.A., Kittock, R.J. and Sequin, B.E. (1974). J. Dairy Sci. 57;89.
Bruce, W. (1956). 3rd Internat. Congr. Anim. Reprod. (Camb) Sec 3 pp 27.
Buckner, P.J., Willet, E.L. and Bailey N. (1954). J. Dairy Sci. 37; 1040.
Bulman, D.C. and Lamming, G.E. (1978). J. Reprod. Fert. 54;447.
Burgess, G.W. and Chenoweth; P.J. (1975). Brit. Vet. J; 131;536.

Campbell, R.C.; Hancock J.L. and Shaw,I.G. (1960) J. Agric. Sci. (Camb.) 55; 91.
Casida, L.E. and Wisnicky, W. (1950). J. Anim. Sci. 9;238.
Cassou, R. (1964). 5th Internat. Congr. Anim. Reprod. Al. Tranto. 540.
Cassou, R. (1967). Elevage, Insem. 97; 3. (ABA 35; 2472).
Cassou, R. (1968). 6th Internat. Congr. Anim. Reprod. A.I. Paris. Summeries; 163.
Cassou, R. (1972). A technique for semen preservation and its application to breed improvement programmes Inst. Med. Vet. Rue clemenceus BP76 L'AIGIE personal publication.
Candrahasen, C., Pattabhiraman, S.R., Venkatswami, V. (1985). Indian J. Anim. Reprod. 6 (2); 142.
Chang, M.C. (1951). Nature (Lond) 168, 697.
Chang, M.C. (1955). Nature (Lond) 175, 1036.
Chang, M.C. and Sneaffer, D. (1957). J. Hered. 48; 107.
Chang, M.C. (1959). In Illoyd C.W. (Ed) "Recent progress in Endocrinology of Reproduction, New York Acadmic Press.
Chauvan, R.A.S., Bhandari, N. and Mathew, A. (1981). Livestock Adviser 6 : 13-14.
Chaudhary, R.R. and Sadhu, D.P. (1983). Anim. Breed. Abstr. 54 (2) 825.
Chenne Gowda, H. and Abdulla Khan, C.K. (1975). Mysore J. Agri. Sci. 9; 145-150.
Chinnaiya, G.P., Kaker, S.S. and Ganguli, N.C. (1979). Zentralblatt Fur. Veterinari Medizin A 26 : 402-407.
Chinnaiya, G.P. and Ganguli, N.C. (1982). Cherion 11 (1) 15-19.
Cloudsley, Thomson, J.L. (1961). Rythmic activity in animal physiology and behaviour Chap. 5. Academic Press N. York.
Colas, G. (1975). J. Reprod. ferti. 42 (2); 277.
Coulter, G.H. and Foote, R.H. (1977). J. Dairy Sci. 60 (9); 1428-1432.
Courot, M. (1962). Ann. Biol. Animale. Biochem. Biophys. I.B.I.D. 2; 157.
Courot, M. (1962). Ann. Biol. Animale. Biochem. Biophys. 2;21.
Courot, M. (1967). Arch. Anat. Microscop. Morphol. Exptl., 56;97.
Crabo, B.G., Bower, R.E., Graham, E.F. & Pace, M.M. (1971). Anim. Breed. Abstr. 40;41.
Crabo, B.G., Brown, K.J. and Grahm, E.F. (1972). J. Anim. Sci. 35 (2); 377.
Cumming, J.N. (1954). Tech. Bull. Minn. Agric. Exptl. Sta. No. 212 pp 32.
Cupps, P.T., Laben, R.C. and Mead. S.W. (1953). J. Dairy Sci. 36; 422.
Darin Benneta, Poulos, A., White I.G. (1974). Anim. Breed. Abstr. 42(8) : 8004.
Davenport (1897). Quoted by Polge and Parkes (1952).
David, K. (1935). Acta. brev. neerl. physiol. 5; 85-108.
David, J.S.C; Bishop, M.W.H. and cambrowier, M.J. (1971). Vet. Rec. 89;81
Davies, D.V., Mann. T. and Rowson, L.E.A. (1957). Proc. Roy. Soc. B 147;332.

Davis, H.P., Trimberger, G.W. and Underbjerg, G.K.L. (1940). J. Dairy Sci. 23;532.
Deshpande, L.V. and Janaki Raman, K. (1985). Indian J. Anim. Reprod. 60 (1); 59.
Deshpande, B.R., Hukeri, V.B.; Velhanker, D.P. and Sane, C.R. (1976); Indian Vet. J. 53:561-563.
Dhami, A.J., Mehta, V.M. and Kodagali, S.B. (1987). Indian. J. Animal Science. 57(12);1272-1278.
Dhami, A.J. and Kodagali, S.B. (1988). Indian J. Anim. Reprod. 9 (2) 124-127.
Dhingra, S.O. Hukeri, V.B., Deshpande, B.R., Shah, G.V. and Seth, A.R. (1984). Indian J. Anim. Reprod. 4(2) 57-59.
Dhanda, O.P., Rao, B.R. and Razdan, M.N. (1981). Indian J. Exptl. Biol. 19 (3); 286.
Doicheva, M. and Paguignon (1980). In Testicular development Structure and Function, Raven Press N.Y. U.S.A.
Donaldson, L.E. (1962). Queensland J. Agric. Sci. 20(2); 203.
Dott, H.M. (1959). Proc. Soc. Study of fert. 10;73.
Dumas (1925). Cited by A.S. Parkes in Marshall's physiology of Reproduction Vol. 1 Part II. 3rd Ed. Lougmans Green Ltd.
Dunn, H.O. and Hafs, H.D. (1953). J. Dairy Sci. 36;577.
Dunn, H.O., Bratton, R.W. and Henderson, C.R. (1953). J. Dairy Sci. 36;524.
Dunn, H.O., Hafs, H.D., Buckner, P.J., Young G.F., Conrad, E.O., and Barnhert, C.E. (1959). J. Anim. Sci. 18;1557.
Dutt, R.H., Simpson, E.L., Christien, J.C., Willet, E.L. and Larson G.L. (1954). J. Dairy Sci. 37; 1429-34.
Elmore, R.G., Bierschawal, C.J., Youngquist, R.W. Cantley, T.C., Kesler, D.J. and Garverick. H.A. (1975). Vet. Med. small Anim. Clin. 70;1346.
El-Manoupy, A.A. Sada, A.A., Fattouil, EL. S.M. Abou-Ahmed, M.M. (1986). Zucht. Hygiene 21 (4) 214-19.
Eliasson, R. (1959). Acta. Physiol. Scand. 46 Suppl. 1.
EL. Danasouri, J. (1988). Theriogenology. 29(6). 1343-1346.
EL. Sawaf, S.A., Badaway, A.B.A., EL Wishy, A.W. (1971). Tier Zucht. Biol. 88; 222.
Emmens, C.W. and Martin, J. (1957). Aus. Vet. J. 33; 63.
Emmens, C.W. and Swyer, G.J.M. (1948). J. Gen. Physiol. 32; 121-138.
Ennen. B.D., Berndtson, W.E., Mortimer, R.G. and Pickett, B.W. (1976). J. Anim. Sci. 43 (3) 651-56.
Erb, R.E. and Ehlers, M.H. (1950). J. Dairy Sci. 33; 853-64.
Erb, R.E. and Flerchinger, F.H. (1952). J. Dairy Sci. 35; 881-888.
Erb, R.E. and Flerchinger, F.H., Ehlers, M.H. and Gasner, Fix. (1956). J. Dairy Sci. 39; 326.
Erb, R.E., Garverick, H.A. Rondel, R.D., Brown, B.L. and Callahen, C.J. (1976). Theriogenology 5;227.
Erickson, W.E. and Graham, E.F. (1959). J. Dairy Sci. 42; 520-528.

Fayami, E., Crabo, B.G., and Graham, E.F. (1979). Theriogenology, 12; 13-17.
Flechon, J.E. (1970) J. de Microscopic 9, 221-242.
Flechon, J.E. and Dubois, M.P. (1974). Anim. Breed. Abstr. 44 (7) 3009.
Flick, D.L. Merilan, C.P. (1988). Theriogenology. 29; 1207-1213.
Flipse, R.J. (1960). J. Dairy Sci. 43; 773-76.
Flipse, R.J. and Almquist, J.O. (1956). J. Dairy Sci. 39, 214.
Flipse, R.J., Almquist, J.O. and Hohnson, P.E. (1956). J. Dairy Sci. 39; 60-65.
Foote, R.H. (1958). J. Dairy Sci. 41; 1109.
Foote, R.H. (1976). J. Dairy Sci. 59(11); 2014-2017.
Foote, R.H. and Arriola, J. (1987). J. Dairy Sci. 70; 2642-2647.
Foote, W.D. and Hunter, J.E. (1964). J. Anim. Sci. 23;517.
Foulkes, J.A., Watson, P.A. (1975) J. Reprod. Fert. 43(2) 349-353
Gallaway, D.B. (1964). Acta. Vet. Scand. 5 Suppl. 2.
Ganguli, N.C. and Kaker, S.S. (1980). Reprod. Nutr. Develop. 20 (3A) 593-99.
Gehlout, B.S. and Srivastava, R.K. (1987). Theriogenology. 28(6) 767-772.
Ghannom, S., Alabiley, H and Decb, S. (1966). Internat. J. Fert. 11(3) 306.
Gilmore, L.O. (1952). Dairy Cattle Breeding J.B. Lippin Caff. Co. Philadelphia
Gledhill, B.L. (1966). Ph.D. Thesis, Acta Vet. Scand. 7, 166.
Gokhale, D.R. (1958). Indian Vet. J. 35; 573-581.
Goldbatt, M.W. (1935b). J. Physiol 84; 208-216.
Goldbatt, M.W. (1936a) Biochem. J. 29, 1346.
Goldbatt, M.W. (1933) Chem. Indust. 52; 1056.
Golubeva, E.D. (1970). Sbnauch. Trod. belorussk Del Khoz Akad. 73 : 146-150, 235. (ABA 40 : 1810).
Goy, R.W. and Jackway, J.S. (1962). role of inheretance in determination of sexual behaviour patterns in "Roats of Behaviour" (EL-Blissed) pp. 96-110 Harpar and Brothers N.Y.
Graham, E.F. and Erickson, W.E. (1959). J Dairy Sci. 42; 3654-67.
Graham, E.F., Vegt. D.W. and Fisher, G.R. (1958). J. Dairy Sci. 41; 1553.
Graham, E.F., Crabo, B.G. and Brown, K.I. (1972). J. Dairy Sci. 55 (3) 372.
Graham, E.F., Vezquez, I.A. Schmehl, M.K.L. and Evenson, B.K. (1976). Proc. 8th Internet. Congr. Anim. Reprod. A.I. Cracow. 4; 896-99.
Graham, E.F., Schmehl, M.K.L., and Evanson, B.K. (1978). Proc. 7th Technical Conference on A.I. and Reprod., Medison, Wisc. NAAB Columbia, Missouri 68-73.
Grewel, N.S., Acharya, R.M. and Dhillon, J.S. (1969). Indian Vet. J. 46; 687.
Hafs, H.D., Boyd, L.J. and Cameron, S. (1970). J. Dairy Sci. 33 : 1683.
Hafs, H.D. and Elliot, F.T. (1955). J. Dairy Sci. 36; 811.

Hafs, H.D. (1971). A research and development challange proc. silv. Anniv. Con. NAAR.
Hafez, E.S.E. (1953). Internat. Physiol. Congr. (Montreal); 19.
Hafez, E.S.E. (1968). Reproduction in Farm Animals 2nd Ed. Len and Febiger, Philadelphia.
Hafez, E.S.E. (1968). Behaviour of Domestic Animals Williams and Wilkins Camp. Baltimore.
Hafez, E.S.E. and Bouissou. (1975). The behaviour of cattle in "Behaviour of Domestic Animals" 3rd Ed. Bailliare Tisdell, London, U.K.
Hafez, E.S.E. (1975). In "Reporduction in Farm Animals" Ed. ESE Hafez 3rd Ed. P.24 Philadesphia, Lea & Fabiger.
Hall, J.B. Branton, C. and Stone, E.J. (1959). J. Dairy Sci. 42;1
Hale, E.B. and Almquist, J.O. (1960). J. Dairy Sci. 43; 145.
Hale, E.B. (1966). J. Anim. Sci. 25 Suppl. 36.
Hahn., Cassou, B. and Elbl., K. (1969). Dt. Tierarztl. Wschr. 76;346.
Hancock, J.L. (1952). J. Exptl. Biol. 29, 445.
Hancock, J.L. (1957). Anim. Breed. Abstr. 26; 320.
Hancock, J.L. and Rollinson, D.H.L. (1949). Vet. Rec. 61; 742-743.
Hankiewiez, J., HanKiewiez, K., Hanus, S. and Szaflarski, J. (1964). Enzymologia 28;100.
Harker, J.E. (1964). Diurnal Rythms and Homeostatic Mechanism in "Homeostasis and Feed back Mechanisms" Symp. Soc. Exptl. Biol. No. 16. 283-300. Academic Press N.Y.
Hart, P.S., Mead, S.W. and Regon, W.M. (1946). Endocrinology 39; 221-23.
Hart, P.S. (1950). J. Agric Sci. (Camb) 40; 143-149.
Hartree, E.F. and Mann, T. (1958). Biochem. J. 69; 50.
Hendrickson, B. (1956). Acta Agri Scand. 7;1.
Herman, H.A. and Madden, F.W. (1953). Handbook and Laboratory Manual, Lucas brothers, Columbia, Missouri.
Heuer, C., Nazir, Tahir, M., Crabo, B.G., Bader, M., Shah, M., and Saji, M. (1983). Pakistan, Vet. J. 3 (4) 15.
Heuston, W.D., Monke, D.R., Milburn, R.J. (1988). J. Amer. Vet. Med. Assoc. 192 (6) 766-768.
Hewett, D. C. (1968); Brit. Vet. J. 124 : 342
Hignett, S.L. (1956). Proc. int. Congr. Anim. Repord. A.I. 116.
Hoglund, H. and Pincus, G. (1942). J. General physiol 25, 337.
Hooker, C.W. (1944). Amer. J. Anat. 74; 1-37.
Hoelzer, H. (1951). Dtsch. Tierarztl. Wschr. 58, 47-48.
Holt, A.F. (1952c). Vet. Rec. 64; 818.
Holy, L. (1971). Veterinary. Med. Bull. 16; 501-12. (ABA 40; 3121).
Hube, A., Uribe, H. (1980) 9th Internat. Congr. Anim. Reprod. Al Madrid. Spain (ABA 49 (4) : 1914).
Hukeri, V.B. (1989). Indo-French Jt. Technical Seminar 11 to 13 Feb. pp. 36. Pune.

Hultnas, C.A. (1961). Cited by Hafez E.S.E., Schein, M.W. and E.W. Bank, R. in "Behaviour of Cattle" pp 261. Behaviour of Domestic Animals ed. Hafez, E.S.E.

Hultnas, C.A. (1967). FAO - SIDA PG course on Animal Repord. Stockholm. Sweden. lecture note.

Hunter, R.H.F. (1967). J. Reprod. Fert. 13, 133.

Hunter R.H.F. (1977). European J. obstretics, gynaecology and Reproductive Biology. 7;267.

Hunter, R.H.F. (1980). Proc. 9th Internat. Congr. Anim. Reprod. A.I. Madrid-Spain Vol. 2; 227-233.

Hunter, R.H.F. (1984). J. Reprod. Fert. 72; 203-211.

Hunter, R.H.F., Nichol, R. and Orabtree, S.M. (1980). Reprod. Nutr. Develop. 20; 1869-75.

Hunter, R.H.F. and Wilmut (1984). Reprod. Nutr. Develop. 24; 597-608.

Hurst, V.J. (1953). J. Dairy Sci. 36; 181-184.

Ibrahim. M.A.R., Rehmen, M.A., Totu, B.L. and Abdin, M. (1983). Acta Veterinariya, Hungeria 31 (1-3); 81-85.

Ibrahim S.S., El. Azeb, A.I., Rakha, A.M. and Soliman, F.A. (1985). Proc. Ist World Buff. Congress Vol. 1 Cairo, Egypt. Dec. 27-31.

Igboeli, G. and Rakha, A.M. (1971). J. Anim. Sci. 33; 651.

Ireland, J.J. Roche, J.P. (1983 a) Endocrinology 112:150

Ireland, J.J. Roche, J.F. (1983 b) J. Anim Sci. 57:157

Ireland, J.J. Fogwell, R.L., Oxander, W.D. Ames, K. and Cawley, J.L. (1985) J. Anim Sci 57:764

Ivanov. (1907). Cited in Marshall's Physiology of Reproduction (Ed). A.S. Parkes, Vol. 1 part 2 3rd Ed. Longman Green and Col. Ltd., (1960).

Jackson, P.S., Johnsen, S.T., Bulman, D.C. and Holdsworth, R.J. (1971). Brit. Vet. J. 135;378.

Jacquet, J. (1951). Bull. Acad. Vet. Fr. 24; 429-432.

Jahnel, J. (1938). Cited by Polge and Parkes (1952). in "Marshall's physiology of Reproduction"

Jackubiee, J. (1984). 34th Annual Meet. of European Assoc. for Anim. Prod. Vol. 2 Summaries. (A.B.A. 52 (1-3); 371).

Jani, V.R., Prabhu, G.A. Darashri, H.J. Patel, M.M. and Kodagali, S.B. (1983). Indian J. Anim. Reprod. 4; 30-32.

Jainuddin, M.R. and Santhana Dass (1982). Animal Production and Health in Tropics, Ser dang, Selengor, Malaysia Penerbit University, Pertanian, Malysia, 409-411.

Jaya Raman, E., Rodrick, I.M., Ratnasabapathy, V. (1979). Cherion 8 (3) 153-158.

James, J.F. (1950). N.Z. Soc. Anim. Prod. 84 pp. Cited by Cole, H.H. and Cupps, P.T. (1959) in "Reproduction in Domestic Animals Vol. 2 pp. 78 Academic press N.Y.

Jaskowski, L. and Szule, L. (1969) Polskie Archoom. Wet. 12; 7-23. (A.B.A. (1970) 38 (3) : 2484).

Johnson, P.E., Flipse, R.J. and Almquist, J.O. (1955). J. Dairy Sci. 38; 53 57.

Jondet, R. (1964). 5th Internat. Congr. Anim. Repord. A.I. Tranto Vol. 4; 463.
Jondet, R. (1972). 7th Internat. Congr. Anim. Reprod. A.I. Munnich. Summeries.
Jones, R.C. (1970). J. Dairy Sci. 51 (8) 1288.
Jones, R.C. (1965). Aust. J. Biol. Sci. 18; 887.
Jones, R.C. (1969). Aust. J. Biol. Sci. 22, 983.
Joshi, S.C. Tiwari, R.B. Sahani, K.L. and Rawat, J.S. (1967). Indian Vet. J. 44; 319.
Joshi, S.C. Rawat, J.S. and Roy A. (1968). Indian J. Vet. Sci. 38; 243.
Kaikini, A.S., Pargaonkar, D.R.; Patil R.V. and Dhindorkar, C.V. (1977), Indian Vet. J.; 54(8), 667-672.
Kaira Dist. Coop. Report. (1972). Proc. FAO-SIDA Followup Seminar, Banglore.
Kale, S.N. (1963). Indian Vet. J. 40; 425.
Kaker, S.S. and Anand, S.R. (1984). Indian J. Exptl. Biol. 22 (1); 5-10.
Kalev, G., Hedzidimitrov, P. and Logoraki, D. (1968). Veterinamo Med. Nauk. 5 (8) 95-101.
Kalev, G. Zogerzki, D. and Pavlov., A. (1974). J. Anim. Health and Prod. 2 (1-2) : 1.
Kavani, F.S. and Kodagali, S.B. (1986). Indian J. Animal Reproduction, 1(1) 53-57.
Kolugin, Y.U.V. (1971). Molashno-Myasno. Skotarstov. 26; 53.
Kampschmeidt, R.F., Mayer, D.T. and Hermen, H.A. (1953a). J. Dairy Sci. 36; 733.
Kampschmeidt, R.F., Mayer, D.T. and Herman, H.A. (1953b). Res. Bull. MO. Agric. Exp. Sta. No. 519. pp. 20.
Kandrick, J.W. and Mc Entee, K. (1967). Cornell, Vet. 57;27.
Kiddy C. A. stone W.H. and Casida L.E. (1959) J. Immun. 82; 125.
King, G.J., Hurnick, J.F. and Robertson, H.A. (1976). J. Anim. Sci., 42,688.
Kinney, W.C. (Jr.) and Van Demark, N.L. (1954). J. Dairy Sci. 37 : 650.
Kirton K.T. and Hafs, H.D. (1965). Science 150; 618.
Kirton K.T., Boyd, L.J. and Hafs H.D. (1968). J. Dairy Sci. 51; 1428.
Kleinm, M.O. and Saroka, M. (1941). Amer. J. Obstr. Gynaeco. 42; 497-99.
Knudson, O. (1954). Acta. Patho. Et. Microbiol. Scand. 101 Suppl. 79 pp.
Kobert (1952). Tierarztl, Umsch. 7; 30.
Kodagali, S.B. (1972). Proc. FAO Swedish Followup Seminar, Bangalore.
Koefred, Johnson (1958). Ann. Rep. Inst. Ster. Res. Royal. Vet. Agric. College Copenhagen pp. 1833.
Kozlo, V.B. (1967). Anim. Breed. Asbstr. 37 (1); 389.
Krishnamurti, P.S., Pattabhiraman, S.R., Venkatswami, V. (1983). Cherion 12 (1) 49-51.
Kuefper (1973). Zucht Hygiene (1972). 7 (4) 145-152. (ABA 41 : 2572).

Kulkami, R.S. and Bhosrekar, M.R. (1973). Indian J. Animal Science 43 (3); 183-86.
Kupferschmeid, H. Gaillard, C., Kozak; A (1975). Anim. Breed. Abstr. 43; 3994.
Kurien, M.O. & Madhavan,E (1985); Indian J. Anim. Reprod., 6(2),14-18.
Kust. D. and Jurgens. (1951). Tierarztl. Umsch. 6; 168-169.

Labhsetwar, A.P., Tyler, W.J. and Casida, L.E. (1963). J. Dairy Sci. 46;843.
Lagerlof, N. (1934). Acta. Patho. Microbiol. Scand. Suppl. 19, pp. 254. (Abstr. Vet. Bull. 1934. 4, 780).
Lagerlof, N. (1938). Proc. 13th Internat. Vet. Congr. Vol. 1 : 214.
Lagerlof, Nils. (1939). Proc. 5th Int. Vet. Cong. Copenhagen 609.
Lagerlof, Nils and Boyd, H. (1953). Comell Vet. 43;52.
Lamming, G.E. (1978). In control of ovulation Ed. D.B. Crigton N.B. Haynes, G.R. Foxcraft and G.E. Lamming. P 335 London, Butterworth.
Lamming, G.E. (1980). Proc. IX Congr. Anim. Repord. A.I. Madrid.
Lanc, S.M., Kira Kofe, G.H., Craig, J.V. and Schalles, R.R. (1983). J. Anim. Sci. 57 (5) 1084-89.
Lardy, H.A. and Phillips, P.H. (1941a). J. Biol. Chem. 138, 195.
Lardy, H.A. and Phillips, P.H. (1941b). Amer. J. Physiol. 134; 542.
Lardy, H.A. and Phillips, P.H. (1945). Arch. Biochem. 6; 53.
Larson, B. (1988). Distribution of Spermatozoa in bovine genital tract and Artificial Insemination Ph.D. Thesis Swedish Univ. Agric. Sciences Upsala - Sweden.
Lasley, J.F., Easley, G.T. and McKenzie, P.F. (1942). Anat. Rec. 82; 167.
Lasley, J.F. and Bogard, R. (1943). Res. Bull. Mo. Agric. Exptl. Sta. No. 376 pp. 56.
Laszczka, A. (1964). Acta. Agar. Silvet. Sar. Zootech 4; 81-144.
Leaver, J.D. (1977). Ani. Prod. 25;219.
Lehrman, D.S. (1953). J. Rev. Biol. 28; 387.
Lehrman, D.S. (1962). Cited by Frazer (1968) in "Reproductive Behaviour in ungulates Academic Press London.
Lepatko, M.I. and Tyupina, K.P. (1972). Zhivetnovodstvo 34 (6); 72.
Lepatko, M.I. and Tyupina, K.P. (1974). Anim. Breed. Abstr. 42 (11) ; 4839.
Linford, E., Glover, F.A., Bishop, C., Stewart, D.L. (1977). J. Reprod. Fert. 47 (2) : 283-291.
Linford, E., Stewart, D.L. and Hebert, C.N. (1977). Brit Vet. J. 133; 9-16.
Little (1919). Cited by Mershall's Physiology of Reproduction. Edited by Parkes, A.S. (1960) Vol. I Part II. 3rd Ed. Longmens Green Co.
Lokhande S.M., Patil V.H., Mahajan, D.C., Phadnis Y.P., Humblot, P. and Thibier, M. (1983) Theriogenology 20 (4) 397-406.
Lokhande S.M., Inamdar, D.R., Joshi, B.M., Bhosrekar, M.R., Humblot. P and Thibier, M. (1984) Rev. Elev. Med. Vet. Paystrop. 37 (1) 73-78

Lotthammar, K.H., Cooke, B.C., Friesecke, H. (1978). Proc. Roche symposium Lond. Basal Hoffmann La Roche.
Lovelock, J.E. and Biges, C. (1954). Biochem. J. 58; 618.
Lovelock, J.E. and Bishop, M.W.H. (1959). Nature (Lond). 183; 1394.
Lorton, S.P.; Sullivan, J.J.; Bean, B. Kaproth, M; Kellgren, H. and Marshall, C. (1988a). Theriogenology 29(3), 593-607.
Lorton, S.P., Sullivan, J.J., Bean, B., Kaproth, M., Kellgren, H. and Marshall, C. (1988b). Theriogenology 29(3), 609-614.
Ludwick, T.M., Olds, D. and Carpenter, M. (1948). J. Dairy Sci. 31; 677.
LutwakMann, C. (1975). Biol. Chem. Biophys. Acta. 58; 637.
Luyet, B.J. and Hodapp E.L. (1939). Anim. Breed. Abstr. 1; 72.
Mahajan, S.C. and Sharma, U.D. (1965). Indian J. Dairy Sci. 16; 15.
Mahamoud, M.F., Ragab, M.R., Yousuf, A.A., Dowidor, M.F. and EL Sherbini, S.C. (1986). Indian Vet. J. 63 (5); 393-396.
MacPherson, J.W., Penner, P. and King, G.J. (1974) Canadian J. Anim. Sci. 54(1) : 47-48.
Mac Pherson, J.W. (1954). Canadian. Comp. Med. 18; 323-325.
Manjunath, S. (1979). Studies on relative efficacy of FVT 300 for an oestrum in white cattle recovered from Foot and Fouth. M.V.Sc. Thesis, Tamil Nadu Agricultural University.
Mann, T. and Walton, A. (1953). J. Agric. Sci. 43; 343-347 (A.B.A. 22 : 122).
Mann, T. (1948a). J. Agric. Sci. 38; 325.
Mann, T. (1948b). "Biochemistry of Semen" London, Methuen & Co., Ltd., N.Y. pp. 240.
Mann, T. (1951). Mammalian semen; Composition. The metabolism and survival. Symp. Biochem. Soc. No. 7 : 11.
Mann, T. and White, I.G. (1956). Nature (Lod) 178; 142.
Mann, T. and White, I.G. (1957). Biochem. J. 65 : 634.
Marshall, F.H.A. (1942). Biol. Rev. 17 : 68-90.
Marshall, F.H.A. (1956). In Marshall's Physiology of Reproduction (Ed). A.S. Parkes Vol. 1. Part 1. pp 1 to 42.
Margolin, S. (1943). J. Dairy Sci. 26; 756-57.
Matharoo, J.S. and Singh, M. (1980). Zeatralblatt fur veterinar Medizin A 27 : 385.
Mathew, A. (1988). 2nd Indo-Danish Seminar, Anand 21-24 March 1988.
Mayer D.T. and Lasley, J.F. (1945). J. Anim. Sci. 4 : 261-269.
MC Carthy, J.E., Stepita, C.T., Johnson, M.B. and Killien, J.A. (1927). Proc. Soc. Exptl. Biol. N.Y. 25 : 54.
Mc Entee, K. (1971). Proc. US ISA 66 Ann. Meet. pp. 160. cited by Roberts S.J. (1961).
Mcfee, A.F., Boyd, L.J., and Swanson, E.W. (1958). J. Dairy Sci. 41; 732.
Mc Gregor, R. (1941). Vet. Rec. 53; 443.
Mc Gregor, R. (1950). Bull. Anim. Behav. 1 (8) : 21-24.
Meacham, T.N., Cunha, T.J., Warnick, A.C., Hentges, J.F. (Jr.) and Hargrave, D.D. (1963). J. Anim. Sci. 22 (1) : 115.

Meding, J.H. (1954). Aarsberctu. Inst. Sterilitets. Forsk Kavet. Og Landbonjsk. (Kbn) 237.
Melampy, R.M., Cavazos, L.F. and Porter, J.C. (1952). J. Dairy Sci. 35 : 140.
Menge, A.C., Typer, W. J., Stone, W. H. And Casida, L. E. (1960) J. Anim. Sci 19; 1330.
Miller, W.J. and Van Demark; N.L. (1954). J. Dairy Sci. 37; 45-51.
Milavanov, V.R. (1934). Principles of Artificial Insemination Leningard. Seljhozgiz; Moscow, 191.
Milk Marketing Board. England and Wales (1957). : A Report.
Miyamoto, H. and Nishikawa, Y. (1979). Japanese J. Zootechnical Sci. 50 (9); 601-608.
Morrow, D.A., Roberts, S.J. Mc Entee, K. and Gray, H.F. (1966). Cornell Vet. 59;173.
Morton, D.B. (1975). J. Reprod. Fert. 45 : 375-378.
Murdisk., R.W. (1958). Amer. J. Vet. Res. 19 : 247.
Musgrave, S.D. and Heath, A.L. (1957). J. Dairy Sci. 40 : 1389-90.
Nagase, H., Niwa, T. and Yameshita, S. (1963). Japanese, J. Anim. Reprod. 9 : 78.
Nagase, H. and Graham, E.F. (1964). 5th Internat. Congr. Anim. repord. Al. Vol. 4 : 387. Tranto
Nagase, H and Niwa, T. (1964). 5th Internat. Congr. Anim. Reprod. A.I. Vol.4 : 410.
Nakano, H (1930). M.H. aus. der. Med. Akad. Zu. Kyoto. 4, 174-198.
Narsimhan, R.S., Krishna, A.R. and Syed Abdul Quayam. (1970). Indian Vet. J. 47 (1) : 19.
Narsimha Rao, A.V. (1974). Indian Vet. J. 51 : 74-75.
Nelson, L. (1958). Biochem. Biophys. Acta. 27 : 634.
Nelson, L. (1962b). In Bishop D.W. (Ed) "Spermatozoan motility" Washington Amer. Assoc. Adv. Sci. Pub. No. 72. pp 171.
Nelson, L. (1962a). Biol. Bull. Woods Hde. 123 : 468.
Neumannwarner, J. (1980). Archivos. de. Medicina Veterinaria, Chile. 12 (2) : 283.
Nishikawa, Y., Waide, Y. and Of Suki (1955). Bull nat. Inst. Agric. Sci. (Chiba) G. No. 11, 67-71.
Norman, C. Brotoff, A, Dunbar, R.S. (Jr.) (1956). Anat. Rec. 125 : 632.
Norman, C., Johnson, C.E. and Porterfied, I.D. (1958a). Anat. Rec. 131 : 585 (ABA 27 : 1352).
Norman, C. Johnson, C.E. and Dunbar, R.S. (Jr.) (1958b). J. Dairy Sci. 41 : 1803.
Norman, C., Joshi, N.M., Khem, A. and Rai, A.V. (1968). 6th Internat. Congr. Anim. Reprod. Al. Paris Summaries 193 (ABA 37 : 409).
O'Conner, L.K. and Smith, G.E. (1959). J. Agric. Sci. 53; 354-57.
O'Dell, W.T. and Almquist, J.O. (1954). J. Dairy Sci. 37 : 652.
OH, Y.K. and Im, K.S. (1965). Anim. Breed. Abstr. 35 (2) : 1404.
Olds, D. and Van Demark, N.L. (1957). Amer. J. Vet. Res. 18 : 603.

Oloufa, M.M., Sayed, A.A. and Badreldin, A.L. (1959). Indian J. Dairy Sci. 12; 10.
Olson, H.H. and Peterson, W.E. (1951). J. Dairy Sci. 34 : 489-490.
Otravant, R. (1953c). Comp. Rend. Soc. Biol. 147 : 1552-56.
Oser, B.L. (1965). Hawk's Physiological Chemistry. MC Grew Hill Book Co. N.Y.
Pace, M.M. and Graham, E.F., (1970). Biol. Reprod. 3; 140.
Pace, M.M. Sullivan, J.J., Elliot, F.L., Graham, E.F. and Coulter, G.H. (1981). J. Anim. Sci. 53 (3) 693.
Pandit, R.K., Srivastava, R.K., Mittal, V.P. and Patel, M.R. (1977). Indian Vet. J. 54; 249-52.
Parkes, A.S. (1956). Pleanary Pap. 3rd Internat. Congr. Anim. Reprod. (Camb) 69-75.
Patterson, D.J.; Kiracofe, G.H.; Stevenson J.S. and Corah, L.R. (1989) J. Anim. Sci. 67; 1895-1906.
Pavitran, K., Vasant, J.K., Rao, M.B. and Anantkrishnan, C.P. (1972). Indian Vet. J. 49 (2); 1125.
Pellarzi, A. (1975). Rivista di Zoo Tecnia e veterinaria 4; 361-66. (ABA 44; 155).
Pendse, M.D.; Kulkarni, P.D. and Sohoni, A.D. (1977), Indian Vet. J., 54(1), 69-73.
Peroshin, V.P. and ObSK Alov, V.P. (1976). Anim. Breed. Abstr. 44 (7) : 3167.
Philips, P.H. and Lardy, H.A. (1940). J. Dairy Sci. 23; 399.
Philips, R.W. and Andrews F. N. (1936) Massachusetts Agri. Exptl. Sta. Bull.331 16 pp.
Pillai, G.P.V. (1980). Studies on anoestrum in cross bred cattle. M.V.Sc. Thesis, Kerala Agricultural University.
Place, V. and Kasde, J. (1961) Vet. Bull. 31 : 481
Platov, E., Olvadis, R., Saab. S.F.A. (1977). Anim. Breed. Abstr. 45 (3) 1311.
Polge, C. Smith, A.V. and Parkes, A.S. (1949). Nature (Lond) : 164; 666.
Polge, C. & Rowson, L.E.A. (1952). Vet. Rec. 64; 851.
Polge, C. and Jakobson, F. (1959). Vet. Rec. 71 : 928.
Porwal, M.L., Khan, F.H. and Karandikar G.W. (1972). Indian Vet. J. 219 (5); 491.
Prabhu, S.S. and Bhattacharya, P. (1951). Indian J. Vet. Sci. 21; 257.
Prabhu, S.S., Guha, S. and Bhattacharya, P. (1953). Indian J. Vet. Sci. 23; 175-184.
Prabhu, S.S. and Bhattacharya, P. (1954). Indian J. Vet. Sci. 24; 35-50.
Prabhu, S.S. (1956). Indian J. Vet. Sci. 26; 21-33.
Raja, C.K. S.V. and Rao, A.R. (1982). Indian J. Animal Science 52 (12) 1241-43.
Rajamanan, A.H., Graham, E.F. and Schmehl, M.H.L. (1971). A.I. Digest. 19 (4) : 16.

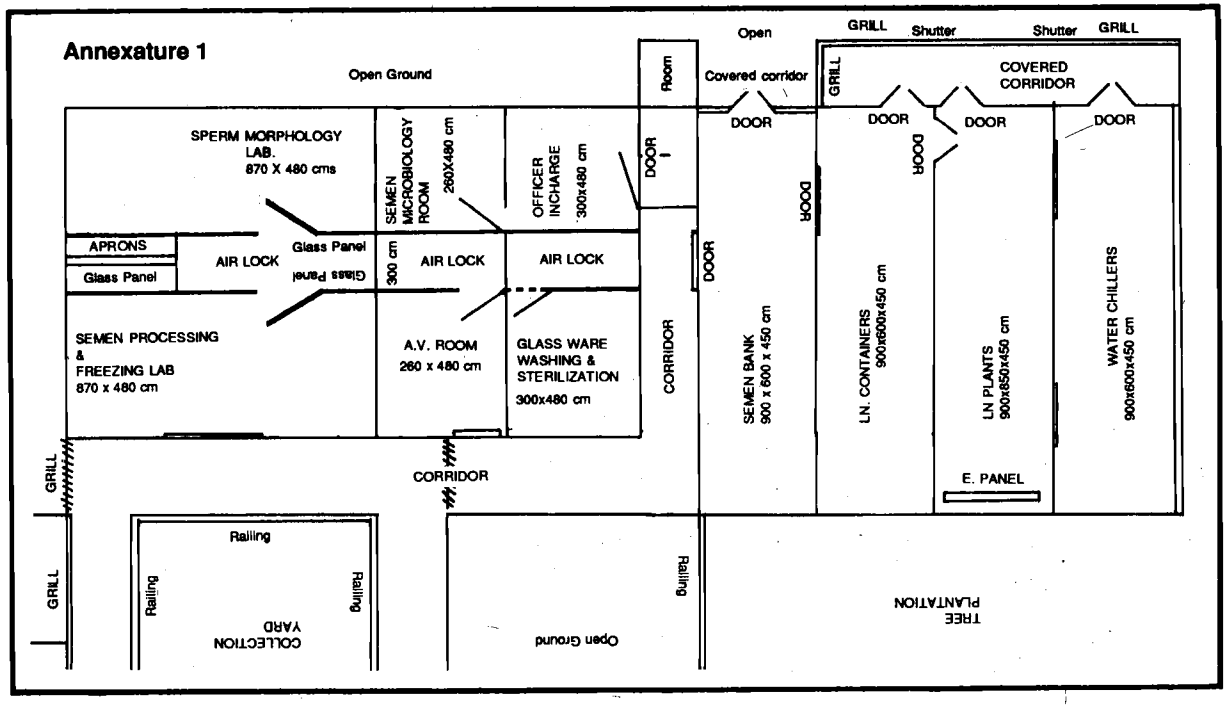
Rakes, J.M. and Stallcup, O.T. (1956). Science, 123; 224-225.
Rao, A.R. (1971). Ph. D. Thesis, Stockholm. Sweden. Roy. Vet. College.
Rao, B.R. and Pandey, J.N. (1977). Indian J. Anim. Sci. 47 : 469-72.
Rao, R.N. and Rao, A.R. (1975). Indian Vet. J. 52 (12); 889-900.
Rendenz, E. (1930). Pro. 2nd Internat. Congr. Sex Res. (Lond).
Rendenz. E. (1933). Biochem. J. 257 : 234.
Reitman, S. and Frankel, S.C. (1957). Amer. J. Clin. Path. 28 : 56.
Rikmanspoel, R. and Van Herpan, G. (1957). Phys. in Med. and Biol. 2 (1) : 54-63.
Roberts, S.J. (1953). Cornell Vet. 43 : 380.
Roberts, S.J. (1971). Veterinary obstretics and genital diseases. Published by the author Edwards Brothers Inc. Arbor/Michigan.
Rodriguez, O.L. Berndtson, W.E. Ennen, B.D. and Pickett, B.W. (1975). J. Anim. Sci. 41 (1); 179.
Roine, K. Solonilni, H. (1978). Acta Vet. Scand. 19,354.
Rojas, M.A., Dyer, I.A. and Cassatt, W.G. (1965). J. Anim. Sci. 24-664.
Rollinson, D.H.L. (1951a). Brit. Vet. J. 107; 203-214.
Rostand, J. (1946). C.R. Acad. Sci. (Paris) 222; 1524.
Rothschild, L. (1950). J. Agric Sci 40 : 82-83.
Rothschild, L. (1950b). J. Exptl. Biol. 26 : 388-395.
Rothschild, L. (1953). J. Exptl. Biol. 30 : 178-179.
Rothschild, L. (1948). J. Exptl. Biol. 25 : 219-226.
Roussel, J.D., Pinero, M.A. and Patric, T.E. (1972). A.I.Digest. 19 (3) : 14.
Rowan E. (1926). Proc. Baston Soc. Natu. Hist. 38; 147-189.
Roy, A. and Bishop, M.W.H. (1959). Nature (Lond) 174; 746-47.
Ruggs, O.D., Berndtson, W.E., Mortimer, R.G. and Pickett, B.W. (1977). J. Anim. Sci. 44 (2) : 266-270.
Saacke, R.G. and Amann, R.P. (1966). J. Anim. Sci. 25 (3) : 929.
Saacke, R.G., Amann, R.P. and Marshall, C.E. (1968). J. Anim. Sci. 27(5); I391.
Saacke, R.G., Almquist, J.O. and Flipse, R.J. (1956), J. Dairy Sci., 39; 90-96.
Saha, S.K. and Singh, R.B. (1958). J. Dairy Sci. 41; 1633-35.
Sakale, J. Turecek, K. Mullerova, Z and Senweraltner, H. (1961) Vet. Bull. 32 : 670
Salam Daudu, C. and Shoyinka, V. (1983). Brit. Vet. J. 139; 487-490.
Salisbury, G.W; Fuller, M.K. and Willet; E.L. (1941). J. Dairy Sci. 24; 905-910.
Salisbury, G.W. and Cragle, R.G. (1956). Paper III. 6 th Internat. Congr. Anim. Reprod and AI. Paris. 2; 1189.
Salisbury, G.W. and Sharma, U.D. (1957). J. Dairy Sci. 40; 677-81.
Salisbury, G.W. and Van Demark, N.L. (1962). Physiology of Reproduction and Artificial Insemination of cattle. W.H. Freeman and Company, San Fransisco & London.
Sangaev, B.E. (1977). Anim. Breed. Abstr. 45 : 752 pp 106.
Sangaev, B.E. & Yurchenko, V.T. (1977). Anim.Breed. Abstr. 45 (2) : 753.

Sanfile, V. (1953). Zoo Profillasi, 7 : 532.
Sapsford, C.S. (1962). Austr. J. Agric. Res. 13; 487.
Saraff, J. and Mixner, J.P. (1955). J. Dairy Sci. 38 : 292-297.
Saxena, V.B., Tripathi, S.S., Verma, H.C. and Rattan Singh (1975). Current Science 45 (4) 154-55.
Schneider, K.M. (1931). Zoo. Cart. 4 ; 349-362.
Schloeth, R. (1961). Z. Tierpsychol, 18; 574-627.
Semakov, V.G. (1963). Vestn. Sal. Nauk. (MOSK) 8 (7); 145.
Sengupta, B.P., Misra, M.S. and Roy, A. (1963). Indian J. Dairy Sci. 16; 150.
Sengupta, B.P. and Roy, A. (1968). Indian J. Vet. Sci. 38-228.
Sengupta, B.P., Singh, L.N. and Roy, A. (1969). Indian J. Anim. Sci. 39; 281.
Sengupta, B.P. and Choube, L.K. (1971). Indian Vet. J. 48; 810.
Sengupta, B.P., Bhela, S.L., Sukhija, S.S. and Tuli, S.K. (1982). 2nd Internat. Conf. World Buff. prod. Casserta Italy IV PP 20-21.
Sergin, N.P. (1957). Austr. Vet. Bull. (Weybridge) 27; 1592.
Settergren, I. (1961). Proc. 4th Internat. Congr. Anim. Reprod. Al. Hague : 752.
Settergren, I. (1962). IX Nord. Vet. Motet Sec. No. 18.
Settergren, I (1963). Bertn. 9 Nord. Vet. Mede. (Kbh). 2 : 559-563 (A.B.A. 32 pp. 483.).
Shaffner, C.S., Henderson, E.W. and Card, C.G. (1941). Poultry Sci. 20 : 269.
Shalash, M.R. (1972). Zootechnica E. Veterinaria 27 (3) : 71.
Sharma, U.D. (1957). Ph.D. Thesis, University of Illinois. Illinois, U.S.A.
Sharma, N.C. (1967). M.V.Sc. Thesis Bombay University. India.
Sharma, S.S. (1977). Ph.D. Thesis, Agra University, Agra, India.
Sharma, N.C. and Sane, C.R. (1972). Indian J. Anim. Sci. 42 (6); 415.
Sharma, R.K., Pareek, P.K. and Vyas, K.K. (1982). Indian J. Anim. Reprod. 2(1); 45.
Sharma, A.R. and Gupta, R.C. (1980). Anim. Reprod. Sci. 3; 217.
Shannon, P. (1972). 7th Internat. Congr. Anim. Reprod. Al Munnich Summeries 268.
Shannon, P. (1968). Proc. N.Z. Sco. Anim. Prod. 28; 23-31.
Shin, S.J., Lein, D.H., Patten, V.H., & Ruhnke, H.L. (1988) Theriogenology 29 (3); 577-591
Signoret, J.P. and Dumensil, Du; Buissou, F. (1962). 4th Internat Congr. Anim. Reprod. 2; 171-75.
Signoret, J.P. and Dumensil du Buissou, F. (1962). Econ. Med. Anim. 3 : 123.
Simmet, L. (1972). Kon fektionierung Von Bullen Sperma in Kunststoffronchrchen nach, ser Land Shutter Metode Tier arztl Umach 27; 88-95.
Simmet, L. (1988). New Filling and Sealing Machine. Personal Publication.
Singh, G., Bhatnagar, M.K. and Pillai, S.K. (1968). J. Anim. Morph. Physiol. 15; 88-94.

Sing, V. and Prakash, A. (1964). Indian, Vet. J. 41; 475-77.
Singh, S.D., Ansari, M.R. and Benjamin, B.R. (1986). Andrologia 18 (1) 33-36.
Skinner, J.D. and Hoaw, A.W. (1966). Anim. Breed. Abstr. 35; 2518.
Smith, J.T. and Herman, H.A. (1954). J. Dairy Sci. 37; 684-690.
Smith, S.E. and Asdell, S.A. (1940). Cornell Vet. 30; 499-506.
Sorensen, E. (1942). Skand. Vettidskr. 32; 358. (ABA 12 : 198).
Sorensen, E. and Hansen, K. (1950). Beretn. Forsgslab. (Kbh) 45 pp. (ABA 19 : 149).
Sorensen, E. and Andersen, S.C. (1956); Paper III Internat. Congr. Anim. Reprod. (Camb) Sec. 1 : 45.
SpallanZani (1803). Cited by Mann, T. in his book "Biochemistry of semen" 1954.
Srivastava, P.N., Adams, C.E. and Hartree, E.F. (1965). Nature. 205; 498.
Srivastava, P.N. and Raza Nasir, M.M. (1968). J. Agric. Sci (Camb) 7; 227.
Stewart, D.L. (1961). 4th Internat. Congr. Anim. Reprod. A.I. Vol. 4 : 975.
Stewart, D.L. (1950). Vet. Rec. 62 : 389-395.
Stolbov, V.M. and Rimanova, L.D. (1984). Anim. Breed. Abstr. 52; 6546.
Strom, S. (1956). Paper 3rd Internat. Congr. Anim. Reprod. (Camb) Sec. 3; 71-72 (ABA 24 : 1674).
Sukhija, S.S. (1984). Ph.D. Thesis. H.A.U. Hissar.
Sukhija, S.S., Sengupta, B.P. and Bhela, S.L. (1983). 4th World Congr. on Human Reprod. Bombay.
Sykes, J.F. and Cole, C.L. (1944). Bull. Mich. St. Univ. Agric. Exptl. Sta. 26 (4) 20-52.
Szerahalyl, A. (1988). Magyar Allatorvosok. Lapja : 43 (2) 85-88.
Szollosi, D., (1962). J. Reprod. Fert. 4;223.
Szollosi, D. (1967). Anatomical Record. 159; 431 - 446.
Szollosi, D. (1975). Mammalian eggs' aging in the fellopian, tube in "Aging gamates" edited by R.J. Blandeu & A.G. Karger Basal Switzerland.
Thacker, D.L. and Almquist J.O. (1953). J. Dairy Sci. 36; 173-180.
Thibier, M. (1972). 7th Internat. Congr. Anim. Reprod. A.I. Munich. (ABA 41 : 2106; 1973).
Tiernney, L.A., Hallford, D.M., Rattle, J.L., Bartlett, D.C. Leighton, E.A. (1982). Proc. Western Section Amer. Soc. Anim. Sci. 33; 114-117.
Tomer, N.S. and Desai, R.N. (1961). Indian Vet. J. 38; 311.
Tomer, N.S., Pande, R. and Desai, R.N. (1964). Indian J. Dairy Sci. 17; 104.
Tripathi, S.S. and Saxena, V.B. (1976). Indian J. Anim. Sci. 46 (1) : 44-47.
Tuli, R.K., Singal, S.P. and Razdan, M.N. (1985). Indian J. Anim. Reprod. 6 (2) : 182.
Tuli, R.K., Bhela, S.L., Sengupta, B.P. and Singal, S.P. (1986). Indian J. Dairy Sci. 39 (3); 323-324.

Upsenskii, A. (1967). Miloch. Myeskotov. (MOSK) 12; 30 (ABA 36 : 1452).
Van Demark, N.L. Mercier, F. and Salisbury, G.W. (1941). J. Dairy Sci. 28; 121.
Van Demark, N.L. and Moeller, A.N. (1951). Amer. J. Physiol. 165; 674-79.
Van Demark, N.L. and Kinney, W.C. (1954). Proc. 7th Annual Con. NAAB Harrisbury. Pennsylvania, PP 192.
Van Demark, N.L. and Hays, R.L. (1955). Amer. J. Physiol. 183; 510-512.
Van Demark, N.L. (1956). 3rd Internat. Congr. Anim. Reprod. (Camb). Plennary PP 80-89.
Van Demark, N.L. and Sharma, U.D. (1957). J. Dairy Sci. 40; 438-39.
Van Demark, N.L. and Bartlett, F.D. (Jr.) (1958). J. Dairy Sci. 41; 732.
Van Euller (1936). Cited by Parkes, A.S. in Marshall's Physiology of Reproduction Vol. 1; Part 2.
Van Rensburg, S.W.J. and devos, W.H. (1962). Onderstepoort J. Vet. Res. 29; 55.
Vantienhoven, A., Salisbury, G.W., Van Demark, N.L. and Hunson, R.G. (1952). J. Dairy Sci. 35; 637.
Vasileva Popava. Jn. Vankova, S. and Pavlov, D. (1969). Proc. Internat. Symp. Varna. Bulgaria, Bulgerian Academy of Sciences Press. 251.
Venketshwar, V. and Jagannadh Rao (1970). Indian Vet. J. 47; 23-29.
Vengust, M., Sanegacnik, J., Vomer, I. Grabljevec., A. Bergant. J. (1981). Veterinarstvo 18 (1) 77-81.
Verma, B.N. (1973). Zootechnica e veterinaria 28 (11/12) 252-56 (ABA 43 : 5783).
Vera, O.N.A. (1980). Archivos de Medicine Veterinaria, Chile. 12 (2) 287-288.
Volosevich, O.P. (1974). Anim. Breed. Abstr. 42 (4) : 3711.
Walton, A. (1958). J. Res. Agric. Soc. (England) 119 : 63-69.
Walton, A and Edwards, J. (1938). Proc. Amer. Soc. Anim. Prod. 31st Annual Meet 254-259.
Ward, R.J. and Moore, T. (1955). Biochem. J. 55, 295.
Warnick, A.C., Meacham, T.N., Cunha, T.J., Roggins, P.E., Hentges, J. F. (Jr.) and Shirley, R.L. (1961). Proc. 4th Internat. Congr. Anim. Reprod. A.I. Hegue Vol. 2; 202.
Warnick, A.C. Fields, M.J., Burns, W.C. and Koger, M. (1969). Proc. 2nd World Conf. Anim. Prod. College Park Md. 429-30.
Watts, T.L. and FuQuay, J.W. (1982) J Dairy Sci 65 (suppl, 1) 230
Watts, T.L. and FuQuay, J.W. (1983) J Dairy Sci 66 (suppl, 1) 262.
Watson, P.F. (1976) Vet. Rec. 197; 12-15
Weitze, K.F. (1973). 7th Internat. congr. Anim. Reprod. AI Munich Summeries; 263.
Wells, M.E., Wonse Fresh, T., Awa, O.A. and Stephens, D.F. (1970). J. Anim. Sci. 31; 67.
Wells, M.E. and Haffley, P.J. (1974). Res. Report. Agri. Exptl. Sta. Oklahoma State University M.P. 92; 267-272.

White, I.G. (1957). Amer. J. Physiol., 189:307.
White, M.B. (1964). Proc. Aust. Soc. Anim. Prod. 5; 33.
Wiggins, H.B. and Almouist J.O. (1975). J. Anim. Sci. 40 (2); 302.
Willett, E.L. and Ohms, J.I. (1957a). J. Dairy Sci. 40; 1324-28.
Willett, E.L. and Ohms, J.I. (1957b). J. Dairy Sci. 40; 1559-1569.
Willett, E.L. and Salisbury, G.W. (1942). Mam. Cornell. Agric. Exptl. Sta. No. 249 PP 45.
Williams, W.W., Green, R.W. and Denhroske, F. (1957). J. Dairy Sci. 40; 621.
Wischart, D.F. and Young, I.M. (1974). Vet. Rec. 95;503.
Wittbank, J.N. Gregory, K.E., Swiger, L.A., Ingells, J.A., Rothlisbarter, J.a. and Koch, R.M. (1966); J. Anim. Sci. 25;744.
Yaseen, A.M. (1971). Alex. J. Agric. Res. 18; (2); 147.
Yaseen, A.M., EL Kamash, M.A. (1972). J. Agri. Sci. (Camb) 78; 363.
Yeates, N.T.M. (1965). The influence of climate in modern aspects of Animal Production PP 89-168. Butterworth-Lond.
Young, I.M. (1989) Veterinary Record 124., 587-88.
Zemjanis, R. (1980). Current therapy in theriogenology, philadelphia; W.B. Sounders.
Ziljcov, N.Z. (1966). Anim. Breed. Abstr. 35 : 2536.
Zondak. B. (1934). Nature 133; 209.
Zvereva, G. and Chuhrly, B. (1972). 7th Internat Congr. Anim. Reprod. A.I. Munnich; Summaries; 473.


Annexature 1
Open Ground
Room
Open
GRILL
Shutter
Shutter
GRILL
Covered corridor
GRILL
COVERED CORRIDOR
DOOR
DOOR
DOOR
DOOR
SPERM MORPHOLOGY LAB. 870 X 480 cms
SEMEN MICROBIOLOGY ROOM 260X480 cm
OFFICER INCHARGE 300x480 cm
DOOR
DOOR
APRONS
Glass Panel
AIR LOCK
Glass Panel
300 cm
AIR LOCK
AIR LOCK
DOOR
SEMEN PROCESSING & FREEZING LAB 870 x 480 cm
A.V. ROOM 260 x 480 cm
GLASS WARE WASHING & STERILIZATION 300x480 cm
CORRIDOR
SEMEN BANK 900 x 600 x 450 cm
L.N. CONTAINERS 900x600x450 cm
LN PLANTS 900x850x450 cm
WATER CHILLERS 900x600x450 cm
CORRIDOR
E. PANEL
GRILL
GRILL
Railing
Railing
Railing
COLLECTION YARD
Open Ground
Railing
TREE PLANTATION

Annexature 2

Semen journal for Deep freezing Date _____ Batch No. _____

Bull	Ejac. vol.	Activity	Conc. ($\times 10^6$)	Motility	Dilution rate	Diluted semen (ml.)	No. of straws	Motility before freezing	Motility after freezing	Storage	Identi-fication	Remarks

Annexature 3 **STUDIES ON SERVING BEHAVIOUR OF EXOTIC CROSSBREED AND BUFFALO BULLS**

Sr. No.	Bull No.	Breed	Body Wt.	Age	Temp of A. V.	Pressure of A. V. No. pumps	False mounts	Preparation (minutes)	Interest (libido)	Erection	Protrusion	Mount	Body position	Type of seeing	Thrust	Reaction time seconds/ minutes	Dismount second/ minutes

Annexsure 4

Indentification :

Bullwise Journal Freezing

NAME : **Reg. No. :** **Colour-Straw –**

BREED : **Colour Ball**

Date	Jour. No.	Ejac. vol. in ml.	Mass Activity	Concn. (X10⁶)	Motility	Dilution Rate	Motility		No. of Straws	Storage	Distribution		Balance	Remarks
							before freezing	after			Freez-Jour No. Date	No. of Straws		

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